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Assessment of nitric oxide synthase activity in vitro and in vivo by gas chromatography–mass spectrometry

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Abstract

A gas chromatographic–mass spectrometric method for the determination of nitric oxide synthase activity is described. The method is based on the gas chromatographic–mass spectrometric measurement of L-[$^{15}N_2$]arginine-derived [^{15}N]nitrite as its pentafluorobenzyl derivative in the negative-ion chemical ionization mode. Application of the method to the analysis of [^{15}N]nitrite formation by purified neuronal nitric oxide synthase revealed K_M values of 3.1 μ*M* by Hanes and 4.6 μ*M* by Lineweaver–Burk for L-[$^{15}N_2$]arginine. The corresponding V_{max} values were 0.204 and 0.228 μmol [^{15}N]nitrite min^{-1} mg^{-1} NOS, respectively. N^G-Nitro-L-arginine and N^G,N^G-dimethylarginine (asymmetric dimethylarginine) were identified by this method as the most potent enzyme inhibitors. Nitric oxide synthase activity was also assessed in vivo by i.v. injection of L-[$^{15}N_2$]arginine in a rat and determination of plasma [^{15}N]nitrite and [^{15}N]nitrate. The assay described in this work allows for accurate, specific and highly sensitive determination of nitric oxide synthase activity in vitro and in vivo.

Keywords: Enzymes; Nitric oxide synthase; Asymmetric dimethylarginine

1. Introduction

Nitric oxide synthases (NOSs, EC 1.14.13.39) are a family of enzymes which catalyze the oxidation of L-arginine to nitric oxide (NO) and citrulline via intermediate formation of N^G-hydroxy-L-arginine [1]. In this reaction, NADPH, Ca^{2+}, calmodulin (CaM), FAD, FMN and tetrahydro-biopterin (H_4B) are required as cofactors [1]. The oxygen atom incorporated into both citrulline and NO has been shown to be derived from molecular oxygen and not from water [2,3]. NOS is inhibited by various N^G-substituted L-arginine analogs such as N^G-nitro-L-arginine (L-NNA), N^G-nitro-L-arginine methylester (L-NAME), N^G-methyl-L-arginine (L-NMA), and N^G,N^G-dimethyl-L-arginine (ADMA, asymmetric dimethylarginine) [4–8]. L-NMA and ADMA are endogenous NOS inhibitors [6,7]. NO plays a critical role in signal transduction pathways in the cardiovascular and nervous systems and is a key component of the cytostatic/cytotoxic function of the immune system [8,9].

Assessment of NOS activity in vitro and in vivo allows for the determination of factors influencing

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NOS activity including pharmacological interventions. The most frequently used assay is based on the NOS-catalyzed conversion of radiolabeled L-arginine to radiolabeled L-citrulline [10,11]. However, this method fails in cells, biological tissues and whole organisms in which only a very small portion of L-arginine is metabolized to L-citrulline via the NO pathway as the greatest part of L-arginine-derived L-citrulline is formed via other pathways such as the urea cycle [12,13]. The limitation of this NOS assay to pure enzyme preparations has led to the development of alternative NOS activity assays applicable to more complex systems [14,15]. Besides problems arising from nonspecific, NOS-unrelated formation of radiolabeled L-citrulline these assays do not provide any information about NOS-catalyzed formation of NO, the actual biologically active reaction product of NOS. Therefore, the aim of the present work was to develop an assay capable of determining accurately and sensitively NOS activity by measuring stable NO metabolites. Nitrite is the sole oxidation product of NO in water in the presence of molecular oxygen [16]. Also, nitrite has been shown to be an oxidative product of NOS-derived NO in vitro and in vivo [1,13]. Furthermore, recent studies from two independent groups using two different analytical methods have shown that plasma nitrite rather than plasma nitrate is a reliable indicator of NOS-catalyzed formation of NO from L-arginine in vivo in humans [17,18]. Considering these facts we developed an assay which is based on the gas chromatographic–mass spectrometric measurement of L-[$^{15}N_2$]arginine-derived [^{15}N]nitrite. The applicability of the method was demonstrated for an isolated neuronal NOS in vitro, and in vivo in the rat.

2. Experimental

2.1. Chemicals

Sodium [^{15}N]nitrite (98% at ^{15}N) and guanidino-[$^{15}N_2$]-L-arginine hydrochloride (98% at both ^{15}N atoms) were purchased from Cambridge Isotope Labs. (Andover, MA, USA). Sodium nitrite was bought from Riedel-de Haen (Seelze, Germany). 2,3,4,5,6-Pentafluorobenzyl (PFB) bromide was obtained from Aldrich (Steinheim, Germany). Pentafluoropropionic (PFP) anhydride was bought from Pierce (Rockford, IL, USA). Toluene and acetone were purchased from Baker (Deventer, The Netherlands). L-Arginine, N^G,$N^{G'}$-dimethylarginine (symmetric dimethylarginine, SDMA), N^G,N^G-dimethylarginine (asymmetric dimethylarginine, ADMA), N^G-nitro-L-arginine (L-NNA), N^G-nitro-L-arginine methylester (L-NAME), N^G-methyl-L-arginine (L-NMA) and all NOS cofactors with exception of NADPH were from Boehringer Mannheim, Germany, H_4B (Dr. B. Schircks Labs., Jona, Switzerland) and CaM (Biomol, Hamburg, Germany) were obtained from Sigma (Munich, Germany). $CaCl_2$ and potassium dihydrogenphosphate were purchased from Merck (Darmstadt, Germany). Dilutions of NADPH, H_4B, FAD, FMN, L-[$^{15}N_2$]arginine and all NOS inhibitors were freshly prepared in distilled water. CaM (1 mg) was diluted in distilled water (1 ml), aliquoted and stored frozen. Purified recombinant rat neuronal NOS was isolated from baculovirus-infected insect cells as described elsewhere [19]. The NOS preparation contained NOS (1.3 mg/ml), EGTA (4 m*M*) and 2-mercaptoethanol (12 m*M*).

2.2. Enzyme assay and nitrite derivatization procedure

Assays with isolated neuronal NOS were performed as follows. NOS cofactors were diluted in phosphate buffer (50 m*M*, pH 7, 5 ml) resulting in the following final concentrations: H_4B, 10 μ*M*; FAD, 5 μ*M*; FMN, 5 μ*M*; $CaCl_2$, 500 μ*M*; NADPH, 0.8 m*M*; CaM, 500 n*M*. Aliquots (400 μl) were spiked with varying concentrations of L-[$^{15}N_2$]arginine and preincubated for 10 min at 37°C. As a rule, reaction was started by addition of NOS at a final concentration of 13 μg/ml. Conditions differing from those mentioned here are described in the respective figure legends. To stop the reaction, aliquots (100 μl) were taken and transferred to 1.5-ml glass vials, that contained an acetonic solution (400 μl) of [^{14}N]nitrite for use as internal standard resulting in a final concentration of 2.5 μ*M* with respect to 100-μl aliquots of the incubation mixture. PFB bromide (10 μl) was added and samples were derivatized by heating at 50°C for 60 min. After cooling to room temperature acetone was removed under nitrogen and reaction products were extracted

with toluene (300 μl) by vortex-mixing for 1 min. Gas chromatography–mass spectrometry (GC–MS) was performed as described below.

2.3. Determination of NOS activity in vivo in the rat

L-[$^{15}N_2$]Arginine hydrochloride (1 mmol) diluted in isotonic saline (2 ml) was injected i.v. into the right jugular vein of a 350-g anesthetized (xylazine, 2 mg/kg body mass; ketamine, 70 mg/kg body mass) male Sprague Dawley rat. Before and at various times after injection, aliquots (about 0.5 ml) of venous blood from the right femoral vein were taken into EDTA monovetes and plasma was immediately prepared by centrifugation (1000 *g*, 5 min) at 2°C. Plasma samples (100 μl) were treated with acetone (400 μl) and PFB bromide (10 μl), derivatized as described above and analyzed by GC–MS (see below). Plasma nitrate was quantified by GC–MS by derivatizing 100-μl aliquots as described [20] with the exception that no [^{15}N]nitrate was added for use as internal standard. Before derivatization with PFB bromide nitrate was reduced to nitrite [20]. Endogenous unlabeled L-arginine and L-[$^{15}N_2$]arginine in rat and human plasma were derivatized to their PFP-methylester derivatives essentially as described [21] using 10-μl aliquots of plasma ultrafiltrate samples (cut-off M_r 20 000). Briefly, plasma ultrafiltrate was evaporated to dryness under a stream of nitrogen, the residue was treated with 2 *M* HCl in methanol (100 μl) and heated at 80°C for 40 min to obtain the methylester (Me) of the amino acids. Following complete evaporation of excess HCl and methanol the residue was treated with pure PFP anhydride (100 μl) and heated at 80°C for 60 min to prepare the *N*-PFP-Me derivatives. Reagent excess was evaporated to dryness and the residue was reconstituted in methylene chloride (300 μl). The study has been approved by the local supervisory committee for studies in animals (Hannover, Germany).

2.4. Gas chromatography–mass spectrometry

GC–MS was carried out on a Hewlett-Packard MS Engine 5989A connected directly to a gas chromatograph 5890 series II (Waldbronn, Germany). A fused-silica capillary column DB-5 MS (30 m×0.25 mm I.D., 0.25 μm film thickness) from J & W Scientific (Rancho Cordova, CA, USA) was used. Helium (70 kPa) and methane (200 Pa) were used as the carrier and the reagent gas, respectively, for negative-ion chemical ionization (NICI). Interface, injector, ion source and quadrupole were kept at 280°C, 200°C for nitrite/nitrate and 250°C for L-arginine, 225°C, and 120°C, respectively. Aliquots (0.5 μl for nitrite/nitrate; 1 μl for L-arginine) were injected in the splitless mode. For nitrite/nitrate analyses the column was held at 70°C for 1 min and then increased to 280°C at a rate of 30°C/min. For L-arginine analyses the column was held at 80°C for 2 min and then increased to 320°C at a rate of 25°C/min. Selected ion monitoring (SIM) of the ions *m/z* 47 ([$^{15}NO_2$]$^-$) for [^{15}N]nitrite and *m/z* 46 ([$^{14}NO_2$]$^-$) for [^{14}N]nitrite was carried out. SIM of the ions [M−2xHF]$^-$ at *m/z* 588 for L-[$^{15}N_2$]arginine and *m/z* 586 for L-arginine was performed.

3. Results

3.1. Gas chromatographic–mass spectrometric analysis of [^{15}N]nitrite and L-[$^{15}N_2$]arginine

The most intense mass fragments in the GC–MS NICI mass spectra of PFB-NO_2 and PFB-$^{15}NO_2$ were *m/z* 46 ([$^{14}NO_2$]$^-$) and *m/z* 47 ([$^{15}NO_2$]$^-$), respectively. The peak area ratio of *m/z* 47 to *m/z* 46 from SIM of 1 nmol of PFB-NO_2 was determined as 0.00456±0.00013 (mean±SD, n=5) at a relative standard deviation (RSD) of 2.9%. The peak area ratio of *m/z* 46 to *m/z* 47 from SIM of 1 nmol of PFB-$^{15}NO_2$ was measured as 0.02868±0.00026 (mean±SD, n=5) at a RSD of 0.9%. The higher value of the peak area ratio of *m/z* 46 to *m/z* 47 from PFB-$^{15}NO_2$ compared with that of *m/z* 47 to *m/z* 46 from PFB-NO_2 is due to the presence of [^{14}N]nitrite in the preparation of sodium [^{15}N]nitrite and in the reagents used. The peak area ratio of *m/z* 46 to *m/z* 47 from SIM of a mixture containing each 0.5 nmol of PFB-NO_2 and PFB-$^{15}NO_2$ was determined as 1.0310±0.0025 (mean±SD, n=5) at a RSD of 0.2% indicating no cross-talk between ion channels *m/z* 46 and *m/z* 47.

In the buffer used to determine neuronal NOS activity nitrite was measured as 258±43 n*M*

(mean±SD, $n=6$) by GC–MS using [^{15}N]nitrite as internal standard. The applicability of [^{14}N]nitrite as an internal standard for [^{15}N]nitrite was tested by generating a standard curve of [^{15}N]nitrite (0–10 μM) in buffer using [^{14}N]nitrite at a fixed concentration (2.5 μM). Linear regression analysis of the data from GC–MS analyses between [^{15}N]nitrite concentration (μM) found (y) and [^{15}N]nitrite concentration (μM) added (x) resulted in the regression equation $y=0.019+1.027x$ ($r^2=0.995$). The limit of detection of the method was determined as 20 amol of [^{15}N]nitrite at a signal-to-noise ratio of 20:1 with a mean precision (RSD) of 4.8%. The limit of quantitation of the method was determined as 4 nM of [^{15}N]nitrite at a mean accuracy of 93% and a mean precision (RSD) of 5.4%. The concentration of nitrite in the buffer used for the determination of the limit of quantitation was 280 nM. All nitrite analyses in the in vitro experiments were performed in duplicate. RSD of nitrite measurements ($n=100$) was below 2.5%. [^{15}N]Nitrite (at 1 μM) was found to remain unchanged in reactions mixtures of neuronal NOS containing all the cofactors except for CaM.

The electron impact ionization (EI, 70 eV) mass spectrum of the *N*-PFP-Me derivative of L-arginine showed a weak molecule cation at m/z 626 (M^+, 15%), a base peak at m/z 507 ([M−C_2F_5]$^+$, 100%), an ion at m/z 119 ([C_2F_5]$^+$, 60%) and other structurally not yet assigned ions (m/z 404, 45%; m/z 392, 75%; m/z 284, 65%; m/z 216, 90%). The NICI mass spectrum of the *N*-PFP-Me derivative of L-arginine showed a base peak at m/z 586 ([M−2xHF]$^-$, 100%) and less intense ions (each at about 15%) at m/z 625 ([M−1]$^-$), 606 ([M−HF]$^-$) and 566 ([M−3xHF]$^-$). The NICI mass spectrum of the *N*-PFP-Me derivative of L-[$^{15}N_2$]arginine showed a base peak at m/z 588 ([M−2xHF]$^-$, 100%) and less intense ions (each at about 15%) at m/z 627 ([M−1]$^-$), 608 ([M−HF]$^-$) and 568 ([M−3xHF]$^-$). These data clearly indicate that the *N*-PFP-Me derivatives of unlabeled and labeled L-arginine are *N*-tri-PFP-Me derivatives.

A standard curve of L-[$^{15}N_2$]arginine (0–1000 μM) in human plasma was prepared without any addition of L-arginine. Linear regression analysis of the data from GC–MS analyses between the peak area ratio of m/z 588 to m/z 586 (y) and the L-[$^{15}N_2$]arginine concentration (μM) added (x) resulted in the regression equation $y=0.011+0.014x$ ($r^2=0.999$). The limit of quantitation of the method for L-[$^{15}N_2$]arginine in untreated human plasma was determined as 2.5 μM at an accuracy of 95% and a precision (RSD) of 17%. The concentration of endogenous L-arginine in this human plasma sample was determined as 72±2 μM (mean±SD, $n=2$). The mean RSD from quadruplicate measurements of L-[$^{15}N_2$]arginine at plasma concentrations above the limit of quantitation was 1.8%.

3.2. Measurement of neuronal NOS activity

At CaM and NADPH concentrations of 50 nM and 800 μM, respectively, NOS-catalyzed [^{15}N]nitrite formation from L-[$^{15}N_2$]arginine was linearly dependent on incubation time for 10 min (Fig. 1). At an NADPH concentration of 800 μM increase in CaM concentration from 50 nM to 500 nM constantly increased [^{15}N]nitrite formation but shortened the linear range from 10 min to 2 min. The use of NADPH at the lower concentration of 200 μM

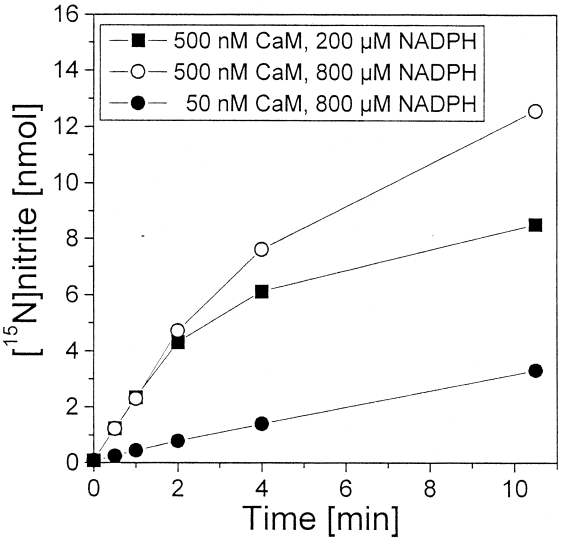

Fig. 1. Dependence of NOS-catalyzed formation of [^{15}N]nitrite from L-[$^{15}N_2$]arginine on incubation time. Samples (1-ml aliquots) in buffer containing L-[$^{15}N_2$]arginine (100 μM) and all the cofactors (NADPH, 0.2 or 0.8 mM; CaM, 50 or 500 nM) were preincubated for 10 min at 37°C, reaction was started by addition of NOS at a final concentration of 13 μg/ml. After the indicated incubation times samples were taken and reaction was stopped by addition of acetone.

revealed also linear increase in [^{15}N]nitrite concentration up to about 2 min but [^{15}N]nitrite concentrations after 4 and 10 min of incubation were lower than those at 800 μ*M* of NADPH. Thus, NOS activity can be determined by the method using incubation times of ≤2 min under optimum cofactor concentrations. NOS activity measured under these conditions was found to depend linearly upon NOS concentrations up to 26 μg/ml (Fig. 2). The mean specific activity of the neuronal NOS used in this study is calculated from this experiment as 94 nmol [^{15}N]nitrite min^{-1} mg^{-1} NOS.

By using different L-[$^{15}N_2$]arginine concentrations (2–100 μ*M*) neuronal NOS activity was determined in a series of experiments performed within 4 weeks. A Michaelis–Menten kinetics for the enzymatic conversion of L-[$^{15}N_2$]arginine to [^{15}N]nitrite was observed (Fig. 3). K_M values determined from the Lineweaver–Burk (A) and Hanes (B) plots were 4.6 and 3.1 μ*M* for L-[$^{15}N_2$]arginine, respectively. V_{max} values were determined as 0.228 and 0.204 μmol [^{15}N]nitrite min^{-1} mg^{-1} NOS, respectively. Characteristic GC–MS chromatograms from these experiments are shown in Fig. 4.

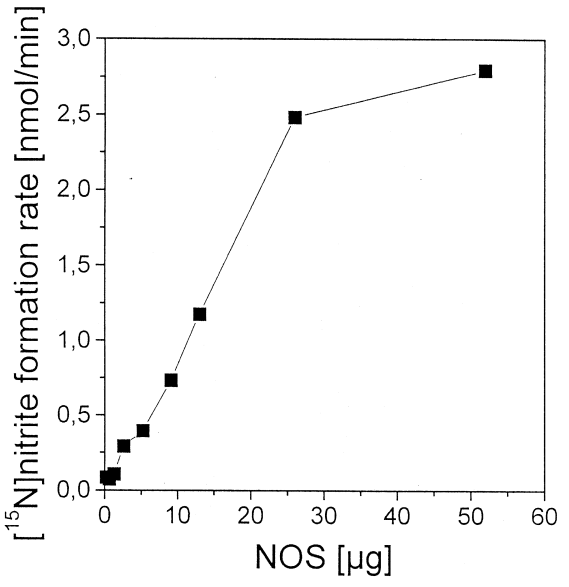

Fig. 2. Dependence of the [^{15}N]nitrite formation rate from L-[$^{15}N_2$]arginine on NOS concentration. All cofactors (NADPH, 0.8 m*M*; CaM, 500 n*M*) and L-[$^{15}N_2$]arginine (100 μ*M*) were preincubated for 10 min at 37°C. Reaction was started by addition of various concentrations of NOS (0.26, 0.65, 1.3, 2.6, 5.2, 9.1, 13, 26 and 52 μg NOS per ml) for 1 min, and stopped by addition of acetone.

Fig. 5 shows that L-NNA, L-NMA, L-NAME, ADMA and SDMA distinctly inhibited NOS-catalyzed formation of [^{15}N]nitrite from L-[$^{15}N_2$]arginine over the whole time range investigated. This figure shows L-NNA as the most powerful inhibitor of neuronal NOS activity followed by ADMA, L-NMA, L-NAME and SDMA. In this experiment, L-[$^{15}N_2$]arginine (20 μ*M*) was converted in the control sample (i.e., no inhibitor) by NOS to produce [^{15}N]nitrite at 6.3 μ*M* which corresponds to a yield of about 30% after 30 min of incubation. HPLC analysis [20] of this sample at this time for L-[$^{15}N_2$]arginine showed complete disappearance of L-[$^{15}N_2$]arginine. We therefore analyzed the reaction mixture for [^{15}N]nitrate by GC–MS using [^{14}N]nitrate (10 μ*M*) as internal standard [20]. [^{15}N]Nitrate was found to be present in the control sample at a concentration of 13.5 μ*M*. These data indicate complete conversion of L-[$^{15}N_2$]arginine to [^{15}N]nitrite and [^{15}N]nitrate at a molar ratio of [^{15}N]nitrate to [^{15}N]nitrite of about 2.1:1. This finding explains the increase in [^{15}N]nitrite formation of only about 1.5 nmol from the second to the 30th min of incubation (Fig. 5). In a further experiment performed under similar conditions using L-[$^{15}N_2$]arginine at a concentration of 100 μ*M* the molar ratio of [^{15}N]nitrate to [^{15}N]nitrite was determined as 2.6:1.

3.3. NOS activity in vivo in the rat

Intravenous (i.v.) injection of L-[$^{15}N_2$]arginine in a rat resulted in a maximum plasma L-[$^{15}N_2$]arginine concentration in the sample taken after 5 min of injection followed by a rapid decrease in its concentration within the next 25 min (Fig. 6A). Thereafter plasma L-[$^{15}N_2$]arginine decreased more slowly (Fig. 6A). Following i.v. injection of L-[$^{15}N_2$]arginine constant increases in the peak area ratio of *m*/*z* 47 to *m*/*z* 46 both for nitrite and nitrate were observed over time indicating formation of [^{15}N]nitrite and [^{15}N]nitrate from i.v. administered L-[$^{15}N_2$]arginine in vivo in the rat (Fig. 6B). In plasma of untreated rats we determined by GC–MS basal concentrations of (mean±SD, n=3) 95±10 μ*M* for L-arginine, 40.4±8.5 μ*M* for nitrate and

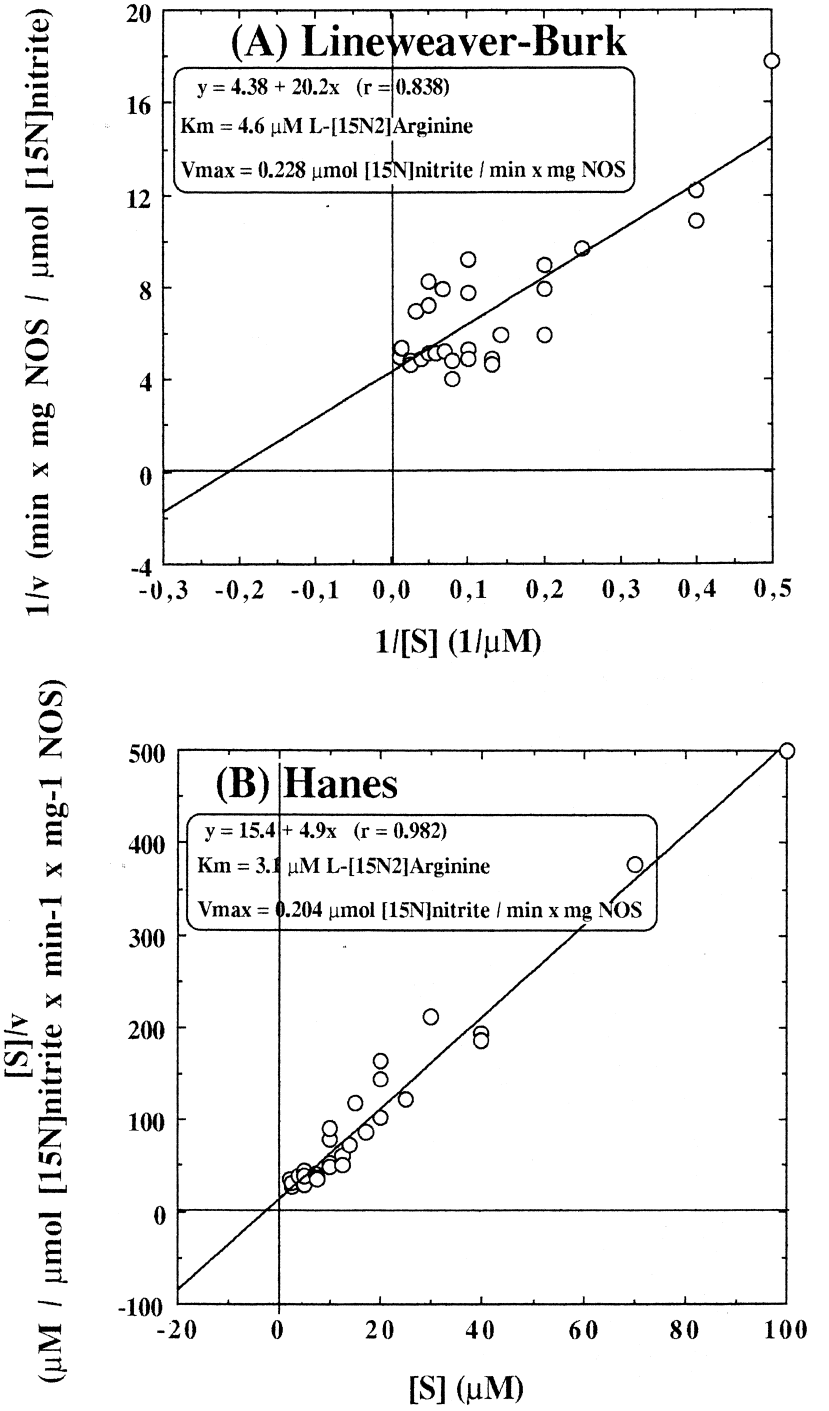

Fig. 3. Lineweaver–Burk (A) and Hanes (B) plots of NOS activity measured at various initial concentrations of L-[$^{15}N_2$]arginine (2–100 μM) preincubated with all cofactors for 10 min at 37°C. Reaction was started by addition of NOS (13 μg/ml) and stopped after 1 min by addition of acetone. [^{15}N]Nitrite was quantified by GC–MS using [^{14}N]nitrite (2.5 μM) as internal standard. To obtain these data 29 measurements were performed within 4 weeks in a series of four separate experiments. S means substrate, i.e., L-[$^{15}N_2$]arginine.

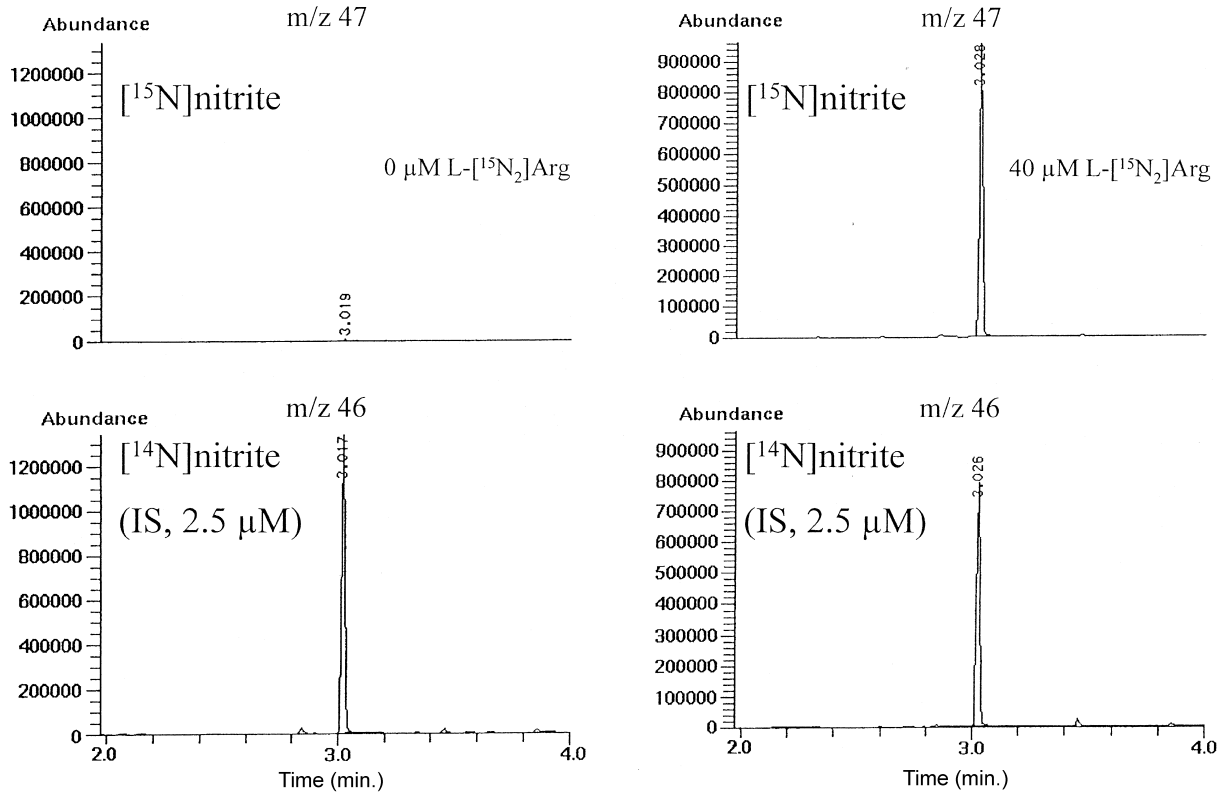

Fig. 4. Partial GC–MS chromatograms from the analysis of NOS-catalyzed formation of [^{15}N]nitrite from L-[$^{15}N_2$]arginine under the conditions described in the legend of Fig. 3 for 0 (left) and 40 μ*M* of L-[$^{15}N_2$]arginine (right). Selected-ion monitoring of *m*/*z* 47 (upper traces) and *m*/*z* 46 (lower traces) was performed.

1.1±0.2 μ*M* for nitrite. Considering these data the concentration of L-[$^{15}N_2$]arginine, [^{15}N]nitrate and [^{15}N]nitrite in rat plasma 150 min after the i.v. injection of L-[$^{15}N_2$]arginine were calculated as 102 μ*M*, 2.2 μ*M*, and 0.05 μ*M*, respectively. These results demonstrate that only a very small portion of i.v. injected L-[$^{15}N_2$]arginine was metabolized via the arginine:NO pathway, and that plasma [^{15}N]nitrite and [^{15}N]nitrate are both metabolites of L-[$^{15}N_2$]arginine in the rat, [^{15}N]nitrate being the major circulating metabolite. The concentration ratio of [^{15}N]nitrate to [^{15}N]nitrite in the rat plasma obtained 150 min after L-[$^{15}N_2$]arginine administration was calculated as 44:1. This is close to the concentration ratio of the endogenous compounds measured in three untreated rats, i.e., 37:1, at the basal state.

4. Discussion

Measuring radiolabeled L-citrulline is the most frequently used assay to determine NOS activity. This assay is rapid but its application is mainly limited to pure enzyme preparations. Moreover, this assay does not provide any information about NOS-catalyzed formation of NO, the actual biologically active reaction product of NOS. Generally, measurement of NO is a difficult undertaking. In pure water that solely contains molecular oxygen, NO is exclusively oxidized to nitrite [16]. Measurement of nitrite could therefore be an alternative method to determine NOS activity in vitro and in vivo [1,13,17,18]. From the methods available to date analysis of nitrite by GC–MS as its PFB derivative is the most powerful approach to accurately and sensi-

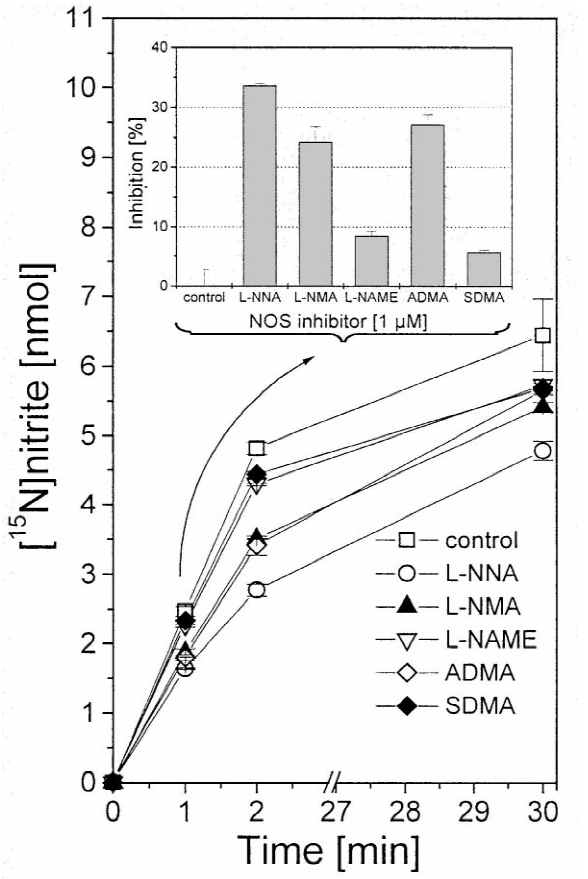

Fig. 5. Inhibition of NOS-catalyzed formation of [^{15}N]nitrite from L-[$^{15}N_2$]arginine by L-NNA, L-NMA, L-NAME, ADMA and SDMA. L-[$^{15}N_2$]Arginine (20 μ*M*) was incubated with all cofactors and NOS (at a final concentration of 13 μg/ml) for the indicated times in the absence (control) and presence of the inhibitor tested (each 1 μ*M*). Reaction was started by the addition of NOS. Insertion shows the extent of inhibition of NOS activity by the inhibitors used. Values are shown as mean±SD from two experiments.

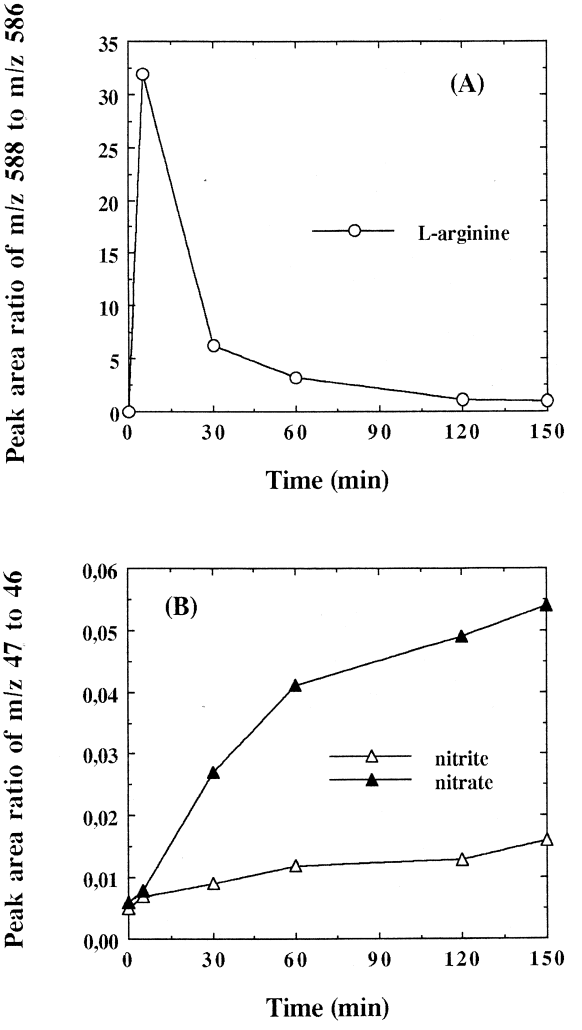

Fig. 6. Relationship between the peak area ratios of *m*/*z* 588 to *m*/*z* 586 for L-[$^{15}N_2$]arginine (A) and of *m*/*z* 47 to *m*/*z* 46 for [^{15}N]nitrite and [^{15}N]nitrate (B) in rat plasma following i.v. bolus injection of L-[$^{15}N_2$]arginine (1 mmol). Prior to and after injection of L-[$^{15}N_2$]arginine venous blood was taken, plasma was generated and ^{15}N-labeled arginine, nitrite and nitrate were determined by GC–MS.

tively measure nitrite in various biological fluids [20]. By measuring L-[$^{15}N_2$]arginine-derived [^{15}N]nitrite in plasma by this method it has been unequivocally shown that the L-arginine:NO pathway is the major source of plasma nitrite in fasted humans [13]. In the present work we extended this method for the determination of [^{15}N]nitrite produced from L-[$^{15}N_2$]arginine by the catalytical action of isolated neuronal NOS and developed a novel assay based on this GC–MS method for the assessment of NOS activity. The present work describes this assay and shows its applicability for the determination of NOS activity of isolated, neuronal, Ca^{2+}-dependent NOS in vitro. This work also shows the method's applicability in vivo in the rat.

In our GC–MS assay, [^{15}N]nitrite and nitrite anions are the real low-molecular-mass species that are formed in the ion-source of the GC–MS instrument, separated by the mass spectrometer and finally

reach the detector. The GC–MS measurement of nitrite as performed in this work, i.e., as its PFB derivative, enables detection of very low [^{15}N]nitrite amounts produced from L-[$^{15}N_2$]arginine by NOS, because of the relatively low natural abundances of ^{15}N (0.37%) and ^{17}O (0.04%), the lack of ^{13}C-atoms in the nitrite anion and practically no cross-talk between ion channels m/z 46 and m/z 47. Since [^{14}N]nitrite was present in the buffer and reagents used we utilized L-[$^{15}N_2$]arginine as substrate for NOS throughout the whole study to demonstrate unequivocally NOS-catalyzed formation of [^{15}N]nitrite from L-[$^{15}N_2$]arginine. To quantify [^{15}N]nitrite by this assay we used [^{14}N]nitrite as internal standard at a concentration of 2.5 μ*M*. The limit of quantitation of the method was 20 n*M* for [^{15}N]nitrite when using [^{14}N]nitrite at 2.5 μ*M* and only 4 n*M* for [^{15}N]nitrite when no [^{14}N]nitrite was additionally added. In this case L-[$^{15}N_2$]arginine-derived [^{15}N]nitrite can be quantitated using [^{14}N]nitrite as the internal standard the concentration of which in the incubation mixtures is relatively constant.

In contrast to other assays, e.g., to those measuring radiolabeled citrulline, our GC–MS method is generally applicable: it allows assessment of NOS activity not only in isolated NOS preparations but also in vivo in whole organisms in which a very low portion of L-arginine is metabolized via the ·NO pathway [12]. Our results from the in vivo study in the rat show circulating nitrite as an endogenous L-arginine-derived species in the rat, and indicate that plasma nitrite is a useful measure for L-arginine-derived ·NO. This has been obtained previously in fasted humans [13]. Our results also show that plasma [^{15}N]nitrate is the major circulating metabolite of administered L-[$^{15}N_2$]arginine in the rat.

Under optimum conditions for cofactors and enzyme concentrations neuronal NOS-catalyzed conversion of L-[$^{15}N_2$]arginine to [^{15}N]nitrite was found to follow the Michaelis–Menten kinetics. The K_M values observed by our assay, i.e., 4.6 μ*M* by the Lineweaver–Burk plot and 3.1 μ*M* by the Hanes plot, are similar to those obtained for neuronal NOS by using assays based on the measurement of radiolabeled L-citrulline [11,22–25]. The V_{max} value determined by the present method is about three times smaller than that obtained for neuronal NOS by measuring radiolabeled L-citrulline or the sum of nitrite and nitrate by the Griess assay [22]. Considering a constant concentration ratio of [^{15}N]nitrite and [^{15}N]nitrate of about 1:2 in our assay, a V_{max} value for the neuronal NOS-dependent formation of [^{15}N]nitrite plus [^{15}N]nitrate of 0.6 μmol min^{-1} mg^{-1} NOS is calculated which is almost identical with that previously obtained for this enzyme using various NOS activity assays [22]. The mechanism(s) by which nitrate was formed in our assay was not further investigated.

Our results show L-NNA as the strongest neuronal NOS inhibitor tested. This finding is in full agreement with results obtained from previous studies on neuronal NOS [24,25]. ADMA has been shown to be an endogenous inhibitor of macrophage and vascular NOS [6]. In the present work, we show for the first time that ADMA is a potent inhibitor of neuronal NOS with a potency comparable to that of L-NNA. Interestingly, we found that ADMA inhibited isolated neuronal NOS activity much more potently than its isomeric SDMA which only possessed a weak inhibitory potency towards neuronal NOS. Stock solutions of SDMA were found by a previously described HPLC method [20] not to contain any ADMA as contamination. Our present results support recent findings of our group showing negative correlation between plasma ADMA concentrations and urinary nitrate excretion (measured by GC–MS) in patients with peripheral arterial occlusive disease [26] or essential hypertension [27]. Thus, accumulation of ADMA in plasma may be a risk factor for the development of endothelial dysfunction and cardiovascular diseases.

In the present study we observed a weak inhibition of NOS by L-NAME. Previous studies have shown that inhibition of NOS by L-NAME requires its bioactivation to the free acid, i.e., L-NNA [28], or results from contaminating L-NNA [25]. HPLC analysis [20] of freshly prepared stock solutions of L-NAME revealed presence of L-NNA to an extent of about 15%. Therefore, the inhibition of NOS activity seen in our study from the use of the L-NAME preparation resulted most likely from contaminating L-NNA.

Recent studies [29,30] have shown that in assays of NOS besides NO and nitrite additional reaction products such as *S*-nitrosoglutathione [29,30] and

hydroxylamine [29] can be formed. In the present work we show that nitrate is an abundant reaction product of isolated neuronal NOS. The mechanisms of the formation of *S*-nitroso compounds and hydroxylamine in NOS assays are still uncertain. We have recently shown that specific quantification of various *S*-nitroso compounds such as *S*-nitroso-*N*-acetylcysteine [31], *S*-nitrosoglutathione and *S*-nitrosocysteine [32], and *S*-nitrosoalbumin [33], and also quantification of hydroxylamine [34] and L-arginine (this study) are best accomplished by GC–MS. Thus, GC–MS is a versatile and reliable analytical approach excellently applicable to assess NOS activity by measuring nitrite and/or nitrate and to quantify almost all participants in the L-arginine:NO pathway as well as their metabolites and/or reaction products. In the present work we show that ADMA is the most potent endogenous inhibitor of neuronal NOS. The endogeneity of ADMA and its possible involvement in various diseases [6,26,27] force further studies to fully characterize this potent inhibitor of NOS. The GC–MS assay described in this work should be useful in such studies.

5. Conclusions

NO produced by the catalytical action of an isolated neuronal NOS in vitro and of various NOS isoenzymes in vivo in the rat is converted to nitrite and nitrate. Nitrite in incubation mixtures of isolated neuronal NOS and in plasma of rats and humans is a useful parameter to determine NOS activity in vitro and in vivo. Unlike the commonly used radiolabeled L-citrulline assay, the assays based on the measurement of nitrite are unrestrictedly applicable to every matrix in vitro and in vivo. Also, these assays provide important informations about the fate of NOS-produced NO. Measurement of nitrite and nitrate by GC–MS as their PFB derivatives is currently the most accurate and sensitive analytical approach that can be utilized to assess NOS activity in vitro and in vivo. The use of L-[$^{15}N_2$]arginine solves the problems originating from ubiquitous nitrite and enables specific assessment of low NOS activities.

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