Serum Levels of \(N^G,N^G\)-Dimethyl-L-Arginine, a Potential Endogenous Nitric Oxide Inhibitor in Dialysis Patients

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Abstract. Nitric oxide (NO) is involved in blood pressure regulation, and its synthesis is inhibited by methylarginines. It has been hypothesized that one of these, asymmetrical dimethylarginine (ADMA), may contribute to dialysis-associated hypertension because it accumulates in the plasma of hemodialysis (HD) patients in a concentration high enough (4 \(\mu\)mol/L) to inhibit NO synthesis in experimental model systems. A precolumn HPLC technique was used to quantify methylarginines (ADMA and symmetrical dimethylarginine [SDMA]) in plasma from HD patients before and after dialysis, from continuous ambulatory peritoneal dialysis (CAPD) patients, and from healthy subjects. Plasma ADMA concentrations were 0.59 \(\pm\) 0.22 (SD) \(\mu\)mol/L in HD patients predialysis (\(n = 19\)) and 0.70 \(\pm\) 0.27 \(\mu\)mol/L in CAPD patients (\(n = 11\)), versus about half of the concentration in control subjects (0.36 \(\pm\) 0.08 \(\mu\)mol/L, \(n = 7\)). The concentrations of SDMA (not an inhibitor of NO formation) were approximately four to five times the ADMA concentrations in both HD and CAPD patients, in contrast to a ratio of 1:1 in the control subjects. Methylarginine concentrations were reduced by 23% and 40% postdialysis, as calculated from ADMA and SDMA values, respectively. No significant correlations were observed between ADMA concentrations, on the one hand, and blood pressure, creatinine and dialysis dose (Kt/V urea), on the other hand. It is concluded that plasma levels of ADMA are considerably lower than those reported earlier in patients treated with HD and also below the levels that hitherto have been thought to have clinical relevance. The role of ADMA in inhibiting NO in dialysis-associated hypertension is questioned. (J Am Soc Nephrol 8: 1437–1442, 1997)

Nitric oxide (NO) is a freely diffusible biological messenger molecule (1) with diverse functions, including blood vessel relaxation. It is synthesized by the conversion of L-arginine to L-citrulline, on demand, by the enzyme NO synthase (NOS) (EC 1.14.13.39). Because the formation of NO is the only means by which it can be regulated, the low availability of its precursor (L-arginine) and/or inhibition of NOS would lead to low NO levels. NOS is competitively inhibited by the arginine metabolites, \(N^2\)-methyl-L-arginine (NMMA) (2) and \(N^2\)-nitro-L-arginine methyl ester (1,3). These metabolites have been administered to experimental animals and human subjects in studies performed to elucidate the physiological and pathological roles of NO in blood pressure regulation. Vascular disease and hypertension are conditions that may include a deficiency of NO production, whereas in shock, inflammation, stroke, and diabetes mellitus, the production of NO may be elevated (4,5).

Vallance et al. (6) reported in 1992 that asymmetrical dimethylarginine (ADMA) and symmetrical dimethylarginine (SDMA) are endogenous substances that are present in normal human plasma and which accumulate in hemodialysis (HD) patients, both reaching plasma concentrations of about 4 \(\mu\)mol/L, each about eight times higher than their respective levels in control subjects. ADMA at this concentration was shown to inhibit NO synthesis in macrophages in vitro, but slightly higher concentrations seemed to be needed for elevation of the blood pressure and stimulation of vasoconstriction in vivo. On the other hand, SDMA appears to have no effect on NO synthesis (6,7). On the basis of these observations, it was proposed that an elevated plasma concentration of ADMA might be implicated in the development of hypertension and atherosclerosis in renal failure patients (6,8). This hypothesis seems very attractive because it provides an explanation of the high prevalence of cardiovascular disease in end-stage renal failure patients and also because it may link hypertension to the adequacy of dialysis treatment, considering that ADMA is a low molecular weight compound that should easily be removed by dialysis. The paper by Vallance et al. (6) has attracted much attention in recent years and has been cited in the literature no fewer than 80 times from 1993 to 1995, as demonstrated by a recent MEDLINE search.

Seeking factors that might be involved in the pathogenesis of hypertension and cardiovascular disease in renal failure patients, we decided to use the method for determination of ADMA described by Vallance et al. (6). Unfortunately, this method did not work for us, primarily because of the low efficiency of methylarginine extraction. A recently developed method, intended for amino acid analysis and based on reversed-phase HPLC (RP-HPLC) was therefore adapted (9). It enabled us to quantify the minute concentrations of ADMA and SDMA present in human plasma. In this communication,
we describe and evaluate the method and present results of the analyses of arginine metabolites in plasma from healthy subjects and patients with chronic renal failure. These results demonstrate that the plasma concentrations of methylarginines, and particularly ADMA, are considerably lower than was earlier reported.

Materials and Methods

Patients

This study was performed on healthy volunteers (n = 7), CAPD patients (n = 11), and HD patients from whom plasma samples were obtained before (n = 19) and after (n = 12) HD. HD treatment was given three times per week. Blood was drawn in the morning after the patients and healthy volunteers had fasted overnight. Antihypertensive drugs were given to 24 patients, i.e., to 80% of the patients. Mean arterial blood pressure (mmHg) ranged from 87 to 143 in the HD patients and from 92 to 128 in the CAPD patients. All CAPD patients had residual diuresis (100 to 1350 ml urine/24 h), but only four of the HD patients had any residual renal function. The protocol of the study was approved by the Ethics Committee of the Karolinska Institute (Stockholm, Sweden).

Chemicals and HPLC

N\textsuperscript{\textcircled{O}},N\textsuperscript{\textcircled{O}}-dimethyl-L-arginine (asymmetrical dimethylarginine; ADMA), N\textsuperscript{\textcircled{O}},N\textsuperscript{\textcircled{O}}-methyl-L-arginine (NMMA), and N\textsuperscript{\textcircled{O}},N\textsuperscript{\textcircled{O}}-dimethyl-L-arginine (di(p-hydroxybenzene-p'-sulphonate; symmetrical dimethylarginine; SDMA)) were purchased from Sigma (St. Louis, MO). ADMA and SDMA, obtained from Calbiochem (San Diego, CA), were also used. All standard chemicals were supplied by various manufacturers and were of the highest grade available. For determination of methylamines, we adapted a fully automated AccQ Tag amino acid analysis HPLC system (Waters, Milford, MA), containing the derivatizing reagent 6-aminquinolyl-N-hydroxysuccinimidyl carbamate (AQC), which has a 100% derivatization yield with amino acids (9) (see also Waters AccQ Tag Operator’s manual). Precolumn derivatization, reactivity to primary and secondary amino acids, and stable derivatives are other characteristics of the method.

The HPLC workstation consisted of Millennium Chromatography Manager (version 2.10) for data acquisition, peak integration, report presentation, and for the system control of the 600S Pump Controller, 616 LC Gradient Pump, 717 Plus Autosampler with a Heating/ Cooling Module, Column Oven, Helium Degasser, 474 Scanning Fluorescence Detector, and 996 Photodiode Array Detector (PDA; Waters). The fluorescence and PDA detectors were used separately or connected in series.

Sample Preparation

Blood was collected in heparinized test tubes and immediately centrifuged (2300 × g; 15 min; 4°C) to isolate plasma. Proteins were precipitated in 3% 5-sulfosalicylic acid (final concentration) and removed by centrifugation (2300 × g; 20 min; 4°C) after 1 h at 8°C. The supernatants produced in this way were filtered through 0.45-μm syringe filters (Millex-HV, Millipore, Bedford, MA) and were stored frozen at −70°C.

The AQC reagent (10 mmol/L; final concentration) included in the AccQ Fluor reagent kit was reconstituted in acetone/titrated and prepared according to the instructions of the manufacturer (Waters). On the day of analysis, the plasma was derivatized with the AQC reagent as follows: 20 μl deproteinized plasma + 60 μl 200 mmol/L borate buffer, pH 8.8, containing 5 mmol/L disodium ethylenediaminetetraacetate (EDTA) + 20 μl reconstituted AQC reagent. In parallel, each sample was also spiked with known concentrations of methylarginines (a mixture of ADMA, SDMA, and NMMA) as follows: 20 μl plasma + 50 μl borate buffer + 10 μl spiking mixture of methylarginines in borate buffer + 20 μl AQC. Typically, the concentrations of each methylarginine in the 10-μl spiking solution added were 5 μmol/L and 10 μmol/L. One minute after mixing, samples were heated for 10 min at 55°C and placed in the autosampler set at 6°C, and 30 μl was injected into the HPLC.

Separations were achieved at 37°C on an AccQ Tag 3.9 × 150 mm, 4 μm, silica-based Nova-Pak C18 reversed-phase column (Waters) specifically designed for use with the AccQ Tag method. Amino acids were eluted in an acetate-phosphate buffer system containing buffer A (19.04 g/L sodium acetate, 970 μl triethylamine, titrated to pH 5.8 with phosphoric acid), buffer B (acetonitrile), and buffer C (ultra-pure 18 megohm water [ELGA]). In the gradient elution program, buffer A was mixed with buffer B to the following percentages of buffer B admixture: 0% (initial), 1% (1 min), 3% (16 min), 6% (25 min), 8% (35 min), and 14% (40 min). Compared with the original work by Cohen and De Antonis (9), the acetonitrile admixture was slowed down after 25 min to prolong the period when the dimethylarginines were eluted. Subsequent samples were injected onto the column after 4 min of washing with 60% acetonitrile (60:40, buffer B:buffer C) and 9 min re-equilibration in 100% of buffer A. Fluorescence was measured at 250 nm (excitation) and 395 nm (emission) and ultraviolet (UV) absorbance (with the PDA detector) at 248 nm.

Quantification of Methylarginines

Quantification was typically done with the fluorescence detector. Control experiments were performed to establish the order of elution and to quantify the methylarginines. Coelution was established for synthetic dimethylarginines (ADMA and SDMA) from two manufacturers (Sigma and Calbiochem). To minimize methodological errors, protein precipitation was the only sample preparation step before amino acid derivatization and HPLC were utilized. Protein precipitation does not affect the recovery of the plasma amino acids routinely analyzed, and we found that this was also valid for the methylarginines. Thus, the same results were obtained in samples spiked with ADMA and SDMA before precipitation of plasma proteins with 5-sulfosalicylic acid and, which was the normal procedure, in samples spiked after this precipitation. This indicates that no unspecified conjugation of methylarginines with plasma proteins occurred.

The PDA detector was used as a variable UV detector at 248 nm, at which wavelength the chromatograms were quantitative and qualitative reflections of the fluorescence chromatograms within the region of interest for this study. The PDA detector and its versatile computer program were also used for studies of peak homogeneity (purity), where the apex spectrum was mathematically compared with the spectra at each time point across a peak. This analysis indicated that the peaks corresponding to ADMA and SDMA were 100% pure. Spectrum matching was performed thereafter to confirm peak homogeneity and identity. During this procedure, peaks were compared to reference spectra stored in the memory of the Waters Millennium PDA library. In contrast to SDMA, which was recognized in all samples, ADMA was not always recognized because of its low concentration.

The AccQ Tag method is linear over a large concentration range (two orders of magnitude) for all amino acids tested (9). The methylarginines were quantified in multiple spiking experiments, each experiment consisting of (1) blanks, (2) 2.5 μM, 5 μM, and 100 μM standards, (3) samples, (4) 2.5 μM and 5 μM spiked samples, and (5)
pooled uremic plasma. Each sample set was analyzed on two occasions. The pooled uremic plasma was a plasma blend from approximately 50 HD patients, yielding 0.59 ± 0.24 μM ADMA and 2.28 ± 0.56 μM SDMA (means from 10 replicate runs). This sample served as a positive control in the experiments.

The purpose of spiking each plasma sample was twofold, i.e., for confirmation that each methylarginine in plasma co-eluted with its synthetic counterpart and for quantification. From each unspiked and spiked sample, linear graphs for ADMA and SDMA, respectively, were drawn. The spiked blanks (standards) produced parallel graphs differing from the sample curves only by the concentrations of ADMA and SDMA in each sample. For the calculation of the ADMA and the SDMA concentrations in each sample, all information from standards and spiked samples was taken into consideration, i.e., the response factors from spiked samples in which the spiking was regarded as an internal standard as well as the response factors from standards. In addition, the sample chromatograms were manually inspected. The recoveries of synthetic ADMA and SDMA added to plasma samples were virtually 100%.

The on-column detection limit of methylarginines in plasma using this HPLC method, in the fluorescence mode, was approximately 0.1 pmol. Because approximately 2 pmol was the lowest amount injected, this limit was well exceeded, and a sample preparation step to concentrate the plasma was not required.

Statistical Analyses
For statistical evaluation, the Mann-Whitney U test, paired t test, and calculation of linear correlation were used as appropriate. P < 0.05 was considered significant.

Results

Chromatography
Endogenous plasma concentrations of methylarginines were quantified fluorimetrically. Raw data showing a fraction of a 45-min chromatographic separation from a typical control plasma is displayed in Figure 1, where ADMA (here 0.32 μmol/L) and SDMA (here 0.38 μmol/L) eluted successively after proline (at 32.8 min) and before an unknown peak. An analogous illustration in a typical uremic patient (here a CAPD patient) is provided in Figure 2. In the figures, unspiked samples are presented in bold print and, on top of these, spiking with ADMA and SDMA is seen. It should be pointed out that the SDMA peaks are higher in the spiked uremic sample (Figure 2) than in the control sample (Figure 1), because uremic samples contain a higher endogenous concentration of this compound.

To verify the fluorescence data, ADMA and SDMA peaks obtained from selected uremic samples were measured with a PDA detector (coupled in series with the fluorescence detector). Peak homogeneity tests and spectral library searches were done on integrated peaks at different wavelengths, and the results of these tests (see Materials and Methods) confirmed the results with the fluorescence detector, presented below.

Plasma Levels of Methylarginines
NMMA was visible in all samples but could only be estimated at approximately one-tenth the ADMA concentrations. Thus, with the procedure used, no differences in NMMA concentrations between control and uremic plasma were detected (data not shown). The ADMA concentrations in samples from the CAPD and HD patients were 0.70 ± 0.27 μmol/L (n = 11) and 0.59 ± 0.22 μmol/L (n = 19), respectively. These levels were significantly increased over the 0.36 ± 0.08 μmol/L (n = 7) control value. The average endogenous SDMA concentrations in healthy subjects were 0.37 ± 0.11 μmol/L (n = 7), i.e., the same as the ADMA concentrations in these samples. In contrast to ADMA, the SDMA concentrations in uremic plasma were considerably increased and estimated at 2.54 ± 0.59 μmol/L (n = 11) in the CAPD and 2.85 ± 0.77 μmol/L (n = 19) in the HD patients. Figure 3 illustrates these results in box-whisker plots showing median, upper, and lower quartiles and range. In conclusion, the ADMA and SDMA concentrations were not affected by the different regimens of

![Figure 1. Chromatographic raw data showing a fraction of a 45-min separation of a typical control plasma and the same sample spiked with known concentrations of methylarginines, as indicated. Blood was processed and run in fluorescence mode on HPLC, as described in Materials and Methods. This plasma sample (bold print) contained 0.32 μmol/L ADMA and 0.38 μmol/L SDMA, respectively, and the spiked samples an additional 2.5 μmol/L and 5 μmol/L ADMA and SDMA, respectively.](image1)

![Figure 2. Chromatographic raw data showing a fraction of a 45-min separation of a typical uremic plasma and the same sample spiked with known concentrations of methylarginines, as indicated. Experimental conditions were the same as those discussed in the legend to Figure 1. The endogenous concentrations of ADMA and SDMA in this CAPD sample were 0.69 μmol/L and 2.84 μmol/L, respectively.](image2)
dialysis treatment. ADMA:SDMA ratios were 1:4 in the patients and 1:1 in the control group. As expected, the plasma concentrations of dimethylarginines were reduced by dialysis, e.g., SDMA by 40%, as presented in Figure 4.

The mean arterial blood pressure (MAP) ranged from 87 to 143 mmHg in the patients. No significant correlation was observed between ADMA concentrations in plasma and MAP (Figure 5). The dose of dialysis (Kt/V urea) (10), predialysis serum creatinine concentration, and SDMA did not correlate with the plasma ADMA levels.

Discussion

With modern fluorimetry, one can determine micromolar concentrations of amino acids separated by HPLC (subpicomolar amounts on-column) without having to concentrate the sample first, provided that the peaks of interest are sufficiently cleansed of impurities. For the quantification of methylarginines, we earlier utilized o-phthalaldehyde (OPA) derivatization, the method most frequently used to determine amino acids in plasma. Unfortunately, we were unable to separate the methylarginines completely from other peaks. However, by using this method, some preliminary results were obtained earlier that essentially agree with the data in the present communication (11).

The present fluorescence method (9) separated the peaks of interest. To estimate the concentrations of methylarginines, various control experiments were conducted, among which the spiking of each sample with synthetic methylarginines was the most important, i.e., calibration was done on an individual sample basis (see Materials and Methods). As illustrated in Figures 1 and 2, this procedure provided chromatograms in which the methylarginine concentrations may, if anything, be overestimated in the event of undetected impurities. Moreover, the sample preparation included no prechromatographic concentration or clean-up step that could selectively alter either ADMA or SDMA.

This communication reveals endogenous plasma concentrations of methylarginines in well-characterized dialysis patients.
The NMMA bevels were roughly estimated at 0.01 to 0.1 μmol/L; a more sensitive method including, e.g., a sample preparation step, would have been required for absolute determinations. However, these data agree with those in the literature reporting much lower NMMA than ADMA levels (6,12).

The average ADMA level of the patients was 0.63 μmol/L, which was significantly higher than in the control subjects, and individual values were below 1 μmol/L in 28 of the 30 HD and CAPD patients studied. The average SDMA concentration was 2.7 μmol/L, which was approximately seven times higher than the SDMA levels in control subjects and four times higher than the ADMA concentrations in the patients. These results, taken together and compared with data from the literature, show differences, particularly in the ADMA values reported. As compared with the study presented here, six-times-higher ADMA concentrations were reported by Vallance et al. (6), including a 1:1 ratio (each approximately 4 μmol/L) of ADMA to SDMA.

Vallance et al. (6) hypothesized that the inhibition of NOS by endogenous ADMA might cause hypertension and immune dysfunction. They based their hypothesis on several experiments demonstrating that ADMA concentrations in the same range as those reported by these investigators are present in uremic plasma and inhibit NO synthesis in vitro and in vivo. They reported that ADMA (but not SDMA) in a concentration of 5 μmol/L inhibited NO synthesis in a preparation of macrophage cytosol NO-synthase in presence of the 30 μmol/L L-arginine, an effect that could be prevented by the addition of 300 μmol/L of L-arginine. They also showed that ADMA caused a dose-dependent increase in tone of precontracted aortic rings, but that practically no effect was observed at concentrations ≤1 μmol/L. In vivo experiments demonstrated that systemic infusion of ADMA in guinea pigs caused ADMA concentrations to rise to about 10 μmol/L and increased systolic blood pressure by nearly 15%. Furthermore, a local infusion of 2 to 16 μmol/min of ADMA in humans caused a dose-dependent fall in forearm blood flow of approximately 30% at an infusion rate of 8 μmol/min. Because forearm blood flow in humans is approximately 50 ml/min (13), this should result in a local plasma concentration of approximately 160 μmol/L, which is more than 200 times higher than the values we reported for uremic plasma. Thus, our finding that the plasma ADMA concentrations in uremic patients are, on average, not higher than 0.63 μmol/L—i.e., about the same level as reported by Vallance et al. (6) in healthy subjects—casts serious doubt on the validity of the hypothesis that ADMA alone is important for the development of hypertension or other biological effects in uremia. The absence of any correlation between serum ADMA levels and MAP, as we report, appears to support this conclusion (Figure 5). However, the cause of hypertension in end-stage renal failure is multifactorial, including overhydration as well as overactivity of several vasoactive compounds (e.g., angiotensin II, catecholamines), which may obscure any relationship between a single factor, such as ADMA and MAP.

Ritz et al. (14) have taken the ADMA hypothesis one step further by suggesting that an accumulation of ADMA in uremic patients in the presence of malnutrition—resulting in reduced levels of arginine (the substrate for NO synthesis)—may explain why there may be a link between malnutrition and increased mortality, the causes of death in maintenance dialysis patients being chiefly cardiovascular diseases. This hypothesis is based on the data of Vallance et al. (6), and it presupposes that malnourished chronic renal failure patients have such reduced arginine levels that the lack of substrate may be of importance. However, most studies show that plasma arginine is not significantly lower than in healthy subjects, whatever the state of nutrition (15–19). In fact, the intracellular concentration of arginine in muscle is higher than 800 μmol/L in all groups of dialysis patients studied (15,16), i.e., considerably higher than the concentration of L-arginine required to offset the inhibiting effect of ADMA in vitro, as reported by Vallance et al. (6).

It was recently reported that plasma from patients with end-stage renal failure inhibits NOS activity in cellular systems in vitro (20). Plasma ADMA levels in five patients, determined by the method of Vallance et al. (6), ranged from 0.4 to 3.2 μmol/L (mean, 1.3 μmol/L), i.e., the values were lower than those reported earlier (6) with only two patients having higher values than those observed in the study presented here. Furthermore, because the levels were lower than those having an effect on NOS in vitro, the authors concluded that ADMA is not the unique molecule responsible for the inhibitory effect but suggested that it may act synergistically with other molecules to inhibit NOS.

In conclusion, our data, which shows plasma ADMA concentrations lower than those that have been found to affect NOS in different model systems, cast serious doubt on the hypothesis that accumulated endogenous ADMA plays a significant role in the development of hypertension in dialysis patients. However, it cannot be excluded that other endogenous inhibitors of the NO pathway may play a role in this respect.

Acknowledgments
This study received financial support from Hospal International, (Lyon, France), Baxter Healthcare Corporation (Deerfield, IL), and Karolinska Institute (Stockholm, Sweden). The authors are grateful to Ms. Monica Eriksson for technical assistance.

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