Symmetrical Dimethylarginine: A New Combined Parameter for Renal Function and Extent of Coronary Artery Disease

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Symmetrical dimethylarginine (SDMA) is the structural isomer of the endogenous nitric oxide synthase (NOS) inhibitor asymmetric dimethylarginine. Whereas the major route of asymmetric dimethylarginine elimination is the hydrolytic degradation by dimethylarginine dimethylaminohydrolase, SDMA is eliminated by renal excretion. SDMA does not directly inhibit NOS but is a competitor of arginine transport. This study showed for the first time that measurement of SDMA can be a marker of estimated GFR and extent of coronary artery disease (CAD). In 97 patients with CAD, SDMA was a marker of estimated GFR. On multiple regression analysis of the CAD parameter stenosis score, SDMA was the only parameter retained. In addition, endothelial cells from the third passage were cultured in medium that contained 70 μmol/L arginine and was incubated for 24 h in the presence of various concentration of SDMA (0, 2, 5, 10, and 100 μmol/L). The levels of nitrate and nitrite in conditioned media, the protein expression of NOS, and the content of reactive oxygen species in endothelial cells were determined. SDMA inhibited dose dependently the NO synthesis in intact endothelial cells, whereas it had no effect on protein expression of NOS. This effect was associated with an increase in reactive oxygen species. Co-incubation with L-arginine but not D-arginine reversed the effect of SDMA on NOS pathway. Our data suggest that SDMA reduced the endothelial NO synthesis, probably by limiting L-arginine supply to NOS. It is concluded that SDMA might be a useful parameter for detecting patients in very early stages of chronic kidney disease and for determining their risk for developing cardiovascular disease.

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Symmetrical dimethylarginine (SDMA) is the structural isomer of the endogenous nitric oxide synthase (NOS) inhibitor asymmetric dimethylarginine (ADMA). ADMA has been shown to correlate with risk factors for coronary artery disease (CAD) such as hypertension (1,2), hypercholesterolemia (3), hyperhomocysteinemia (4–6), insulin resistance (7), age (1), and mean arterial pressure (1). Moreover, ADMA correlates with the extent and the severity of coronary atherosclerosis (8) and is a strong and independent marker of cardiovascular events and mortality in selected patient populations (9–11). Although there is mounting evidence that chronically elevated ADMA may contribute to progression of vascular disease *via* endothelial damage, little attention has been paid to the role of SDMA. Both ADMA and SDMA derive from intranuclear methylation of L-arginine residuals and are released into the cytoplasm after proteolysis (12). SDMA is produced by protein-arginine methyltransferase 5 (PRMT 5) and PRMT 7 (both type II methyltransferases) (13,14). Only ADMA but not SDMA is metabolized by dimethylarginine dimethylaminohydrolase to citrulline and dimethylamine (15). SDMA seems to be strictly eliminated by renal excretion (16,17). A recent study by Fliser *et al.* (18) showed a very close correlation among serum creatinine, GFR (measured by iodothalamate clearance technique), and SDMA. The authors speculated that SDMA, equal to serum creatinine, can serve as a marker of renal function, but SDMA seems to be more than a simple indicator of renal function. In animals, a high-fat, high-cholesterol diet increases SDMA serum levels (19,20) without affecting renal function (20), indicating that cardiovascular risk factors other than impaired renal function can influence SDMA plasma levels. Furthermore, in intensive care patients, elevated SDMA correlated much better with the total sequential organ failure assessment score than ADMA and indicated both renal and hepatic failure (21).

SDMA does not interfere directly with NOS activity as ADMA does, but it is a potent competitor of L-arginine transport (22). Furthermore, SDMA impairs L-arginine uptake from the perfused loop of Henle (23). These data suggest that SDMA

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indeed could inhibit indirectly NO synthesis by limiting arginine availability to NOS.

Our study was designed to determine whether plasma SDMA levels can be a marker of renal function and beyond that can serve as a marker of severity of atherosclerosis found on coronary angiography. Moreover, we investigated the effect of SDMA on intact cultured endothelial cells.

Materials and Methods

Patients

SDMA, ADMA, and L-arginine were measured in 147 patients who underwent elective coronary angiography. Patients with acute coronary syndromes, coronary bypass, or any operation within the last 3 mo before admission were excluded as were patients with chronic kidney disease (CKD) stage 5 according to the Kidney Disease Outcomes Quality Initiative. Two experienced interventional cardiologists independently scored the degree of CAD using a method described by Sullivan *et al.* (24). Briefly, the coronary tree is divided in eight major segments; one score assesses the number and the degree of more circumscript coronary stenosis (stenosis score ranging from 0 to 32), and the other score characterizes the extent of diffuse coronary arteriosclerosis in these segments (extent score ranging from 0 to 100).

Fasting plasma samples of all patients were drawn on the morning before angiography and stored at −70°C. From these samples, levels of L-arginine, SDMA, ADMA, and citrulline were determined as orthophthalaldehyde derivatives by liquid chromatography–mass spectrometry (LC-MS) (25). The within-assay and between-assay variation coefficients were <7.9%. All other parameters were determined using certified methods. GFR was estimated using the Modification of Diet in Renal Disease formula (26). To evaluate the influence of all obtained parameters on SDMA levels, we performed a stepwise multiple linear regression.

Cell Culture Studies

Materials. SDMA and L- and D-arginine were delivered by Sigma (Tautkirchen, Germany). Human umbilical vein endothelial cells (HUVEC) and the cell culture medium that contained 70 μmol/L arginine were obtained from Clonetics/Cambrex (Kerviers, Belgium).

Cell Culture. HUVEC on third passage were cultured in endothelial basal medium that contained 70 μmol/L arginine as described previously (27). After reaching confluence, endothelial cells were treated with SDMA (2, 5, 10, and 100 μM), SDMA plus L- or D-arginine (200 and 400 μM), or vehicle for 24 h as indicated for each experiment. At the conclusion of the 24-h treatment period, the cells and the supernatants were harvested and saved for the following measurements. The total cellular protein was measured using BCA protein assay kit (Pierce, Rockford, IL).

Measurement of Nitrate and Nitrite. The determination of nitrate and nitrite in cell culture supernatants was carried out in accordance with the method described by Tsikas *et al.* (28). In our laboratory, the intraday precision test yields a relative SD of 3.8% for nitrite and 1.3% for nitrate. The interday precision test yields a relative SD of 4.4% for nitrite and 4.2% for nitrate.

Western Blot Analysis of Endothelial NOS (eNOS). Confluent HUVEC that were treated with various concentrations of SDMA for 24 h were lysed with mammalian cell lysis kit (Sigma, Steinheim, Germany). Lysates were cleared by centrifugation for 10 min at 13,000 × *g* at 4°C. Extracted proteins (20 μg) that were separated by 8% SDS-polyacrylamide gels were transferred into nitrocellulose membranes at 100 mA for 80 min by use of PerfectBlue Semi-Dry-Electroblotter transfer system (PEQLAB Biotechologie GmbH, Erlangen, Germany). Nonspecific binding sites were blocked with Roti-Block (Roth, Karlsruhe, Germany) for 1 h at room temperature. After being blocked, the membranes were washed three times in PBS that contained 0.1% Tween for 10 min at room temperature. Immunoblotting was performed at room temperature for 2 h with anti-eNOS antibody at 1:500 dilution (Santa Cruz Biotechnology, Santa Cruz, CA). After being washed three times in PBS that contained 0.1% Tween for 10 min at room temperature, the membranes were incubated with an appropriate secondary antibody conjugated with horseradish peroxidase (1:2000 dilution; Cell Signaling, Frankfurt, Germany). Membranes were developed by using SuperSignal chemiluminescence substrate (Pierce) and quantified by densitometry.

Detection of Oxidative Stress. Dihydrorhodamine 123 (DHR123) was used as a marker for intracellular reactive oxygen species (ROS). The cells were incubated for 20 min at 37°C in the presence of 10 μM DHR123 with gentle agitation. The reaction was stopped by cooling on blue ice for 1 min and subsequent addition of 500 μl of PBS followed by two washing steps. After a final fixation with 1% paraformaldehyde, the cells were analyzed by flow cytometry (Epics XL-MCL; Coulter, Krefeld, Germany). The level of ROS was indicated by mean fluorescence intensities of stained probes *versus* negative controls.

Statistical Analyses

All data are given as mean ± SEM from at least three independent experiments that were performed in duplicate. Statistical significance was tested with repeated measures ANOVA using a LSD *post hoc* test or multiple comparisons (SPSS Software 11.0; SPSS Inc., Chicago, IL). Differences were considered significant at $P < 0.05$.

Results

Patients

Of the 147 patients who underwent coronary catheterization, 50 did not have significant CAD (stenosis score <3). The reason for coronary angiography in these patients was exclusion of structural heart disease before electrophysiologic testing or radio-frequency ablation for paroxysmal tachyarrhythmia. Therefore, these patients represent a well-defined control group without relevant structural heart disease (Table 1).

Compared with control subjects, the 97 patients with CAD were older (61 ± 10 *versus* 55 ± 14 yr; $P = 0.001$) and more frequently had hypertension (61.9 *versus* 30%; $P < 0.001$) and diabetes (17.5 *versus* 4.0%; $P = 0.012$). They had higher levels of Lipoprotein(a) (median 25 *versus* 11 mg/dl; $P = 0.015$) and creatinine (1.35 ± 0.51 *versus* 1.15 ± 0.26 mg/dl; $P = 0.012$) and lower HDL cholesterol (46 ± 12 *versus* 60 ± 24 mg/dl; $P < 0.001$).

Plasma levels of SDMA (0.62 ± 0.14 *versus* 0.74 ± 0.27 μM; $P = 0.004$) and ADMA (0.62 ± 0.12 *versus* 0.66 ± 0.12 μM; $P = 0.049$) were significantly higher in patients with CAD. There were no differences in the calculated ratios of L-arginine/ADMA, which is an indicator of NO production (165 ± 46 *versus* 162 ± 44), and ADMA/SDMA, which is an indicator of dimethylarginine dimethylaminohydrolase activity (0.96 ± 27 *versus* 1.04 ± 0.24).

Parameters that entered the stepwise multiple regression were general descriptive parameters (age and body mass index), typical renal function parameters (GFR, parathyroid hormone [PTH], calcium, phosphate, and calcium phosphate product), and the CAD disease severity parameters (stenosis score,

Table 1. Selected clinical characteristics of patients ($n = 97$) with CAD[a]

Variable	Values	Variable	Values
Age (yr)	61 ± 10	Creatinine (mg/dl)	1.35 ± 0.51
BMI (kg/m^2)	26 ± 4	CRP (mg/dl)	0.87 ± 1.91
Height (cm)	174 ± 8	IL-6 (pg/ml)	8.96 ± 16.77
Weight (kg)	80 ± 12	GFR (ml/min)	71.24 ± 31.25
Uric acid (mg/dl)	7.63 ± 1.91	Ca × P (mg^2/dl^2)	11.13 ± 2.51
Urea (mg/dl)	20.48 ± 10.79	PTH (pg/ml)	49.71 ± 29.53

[a]Values are given as mean ± SD. BMI, body mass index; CAD, coronary artery disease; CRP, C-reactive protein; Ca × P, calcium × phosphate product; PTH, parathyroid hormone.

vessel score, and extent score). Of these 10 parameters, only four were retained in the final model that accounted for >60% of the variance of SDMA values ($r^2 = 0.601$, $P < 0.001$). The four parameters that were retained were the renal function parameters GFR and PTH and the CAD parameters stenosis score and extent score (Figure 1). The final model held the following equation: estimated SDMA (nmol/L) = 899 − 5 × GFR + 2.17 × PTH + 26 × stenosis score − 7 × extent score.

A stepwise multiple regression analysis of the CAD parameter stenosis score was performed in all patients to determine whether renal function or NO inhibitor state may account for the observed regression. Parameters that entered this regression analysis were in addition to SDMA the NO inhibitor state parameter ADMA and the renal function parameters GFR and PTH. Only SDMA was retained in the final model ($r^2 = 0.075$, $P = 0.003$).

Furthermore, to test whether the renal function parameter GFR is determined solely by SDMA measurement, we performed a stepwise multiple linear regression analysis in which among SDMA the CAD scores, ADMA and PTH entered the analysis. Only SDMA was retained in the final model and accounted for more than one third of the GFR variance ($r^2 = 0.355$, $P < 0.001$; Figure 2). The final model held the following equation: eGFR = 124.5 − 74.4 × SDMA (μmol/L), where eGFR is estimated GFR.

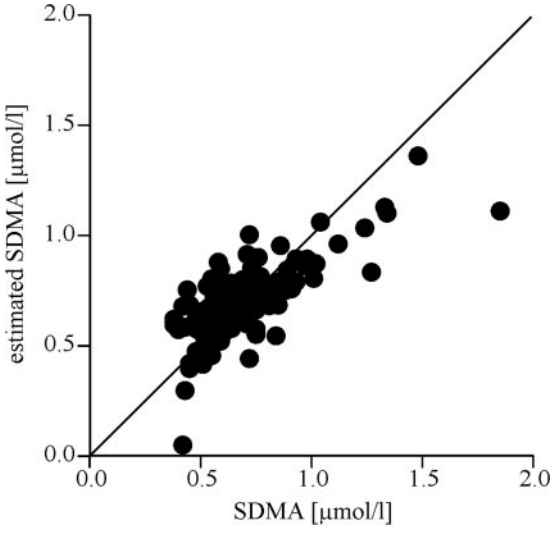

Figure 1. Estimated symmetrical dimethylarginine (SDMA; μmol/L). The four parameters that were retained in the regression model were the renal function parameters GFR and parathyroid hormone (PTH) and the coronary artery disease (CAD) parameters stenosis score and extent score. The final model had the following equation: estimated SDMA (nmol/L) = 899 − 5 × GFR + 2.17 × PTH + 26 × stenosis score − 7 × extent score.

Endothelial Cell Studies

To investigate the effect of SDMA on the NOS system, we cultured endothelial cells in endothelial basal medium that contained 70 μmol/L arginine and incubated them with SDMA (2, 5, 10, and 100 μM) for 24 h. SDMA reduced dose dependently NO synthesis in the conditioned medium (Figure 3A). Western analysis revealed that the expression of eNOS was not altered by SDMA (data not shown). Addition of the NOS substrate L-arginine abolished the effect of SDMA-inhibited NO synthesis, whereas the enantiomer D-arginine was without effect (Figure 3B).

Because the NO synthesis but not the protein expression of eNOS was reduced by SDMA, we hypothesized that this inhibi-

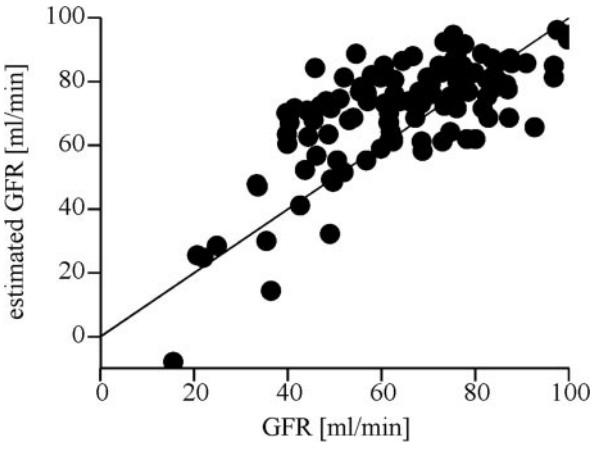

Figure 2. Estimated GFR (eGFR; ml/min). Only SDMA was retained in the final model and accounted for more than one third of the GFR variance ($r^2 = 0.355$, $P < 0.001$). The final model had the following equation: eGFR = 124.5 − 74.4 × SDMA (μmol/L).

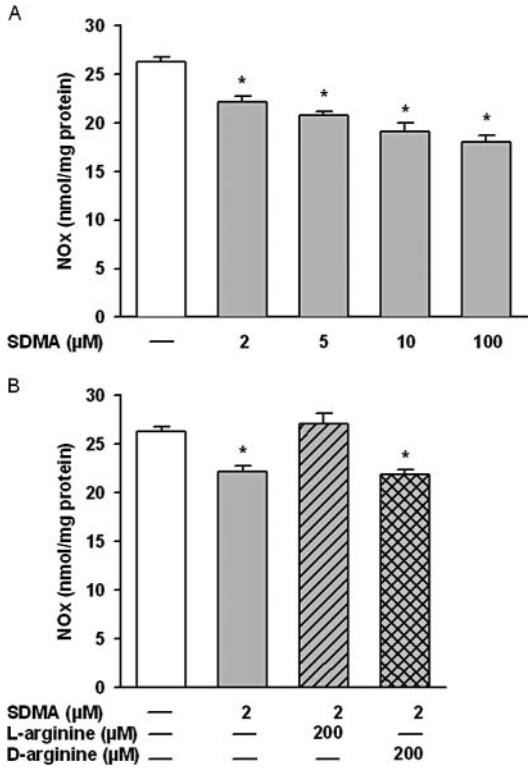

Figure 3. Effect of SDMA on nitric oxide (NO) synthesis in the conditioned cell culture media. Confluent human umbilical vein endothelial cells (HUVEC) were incubated with SDMA or SDMA plus L-arginine or D-arginine. NO metabolites (NOx) concentrations in the medium were measured after 24 h by gas chromatography–mass spectrometry. (A) NOx synthesis in presence of control or SDMA (2, 5, 10, and 100 μM). (B) NOx synthesis in presence of control, SDMA (2 μM), L-arginine (200 μM with 2 μM SDMA), or D-arginine (200 μM with 2 μM SDMA). Values are expressed as nmol/mg protein. Data are expressed as the mean ± SEM of n = 3 different experiments performed in duplicate. *$P < 0.05$ *versus* control.

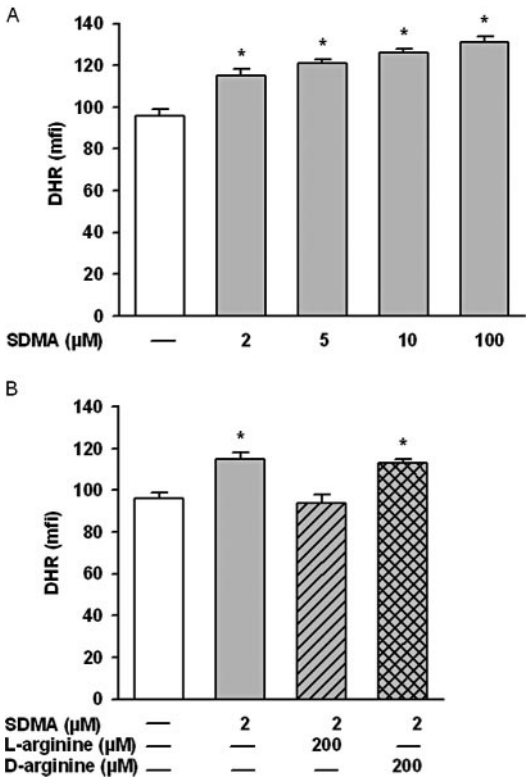

Figure 4. SDMA-reduced NO synthesis leads to increased intracellular oxidative stress. Confluent HUVEC were incubated for 24 h in the presence of SDMA or SDMA plus L-arginine or D-arginine. Endogenous reactive oxygen species (ROS) was measured with dihydrorhodamine 123 (DHR123) using FACS analysis. (A) ROS formation in the presence of control or SDMA (2, 5, 10, and 100 μM). (B) ROS formation in the presence of control, SDMA (2 μM), L-arginine (200 μM with 2 μM SDMA), or D-arginine (200 μM with 2 μM SDMA). Values are expressed as mean fluorescence intensities. Data are expressed as the mean ± SEM of n = 3 different experiments performed in duplicate. *$P < 0.05$ *versus* control.

tion could be associated with an increase in the content of ROS. The endogenous ROS formation was measured by detection of DHR. The intracellular level of ROS was increased significantly in endothelial cells that were incubated with SDMA for 24 h as compared with controls in a dose-dependent manner (Figure 4A). The stimulatory effect of SDMA on ROS production was reversed by co-incubating the cells with L-arginine, whereas D-arginine had no effect (Figure 4B).

Discussion

This study shows for the first time that measurement of the SDMA can be a marker of eGFR and extent of CAD. CKD is a significant public health problem. The worldwide rise in the number of patients with CKD is reflected by the increasing number of people who have ESRD and are treated by renal replacement therapy: Dialysis or transplantation. Patients with CKD even in stages 1 through 4 are in the highest risk group for the development of cardiovascular disease (CVD), and, accordingly, recent guidelines and position statements have defined CKD as a CVD equivalent (29). Hence, evaluation of renal function becomes a means to assess cardiovascular risk. Unfortunately, estimates of GFR from serum creatinine are insensitive even to moderate reductions in GFR and are complicated by considerable interindividual variability as a result of muscle mass, protein intake, age, and gender. Furthermore, obtaining accurate 24-h timed urine collections is not only labor intensive and expensive but also fraught with difficulties for patients. As Levey *et al.* (26) in 1999 proposed that a more accurate method to estimate GFR is calculating GFR from a multiple regression model but as recently presented by Levey *et al.* (30) on behalf of Kidney Disease: Improving Global Outcomes, even practicing nephrologists are hesitant to use it. Therefore, a stable, convenient, and clinically reliable marker of GFR would be desirable. Moreover, traditional cardiovascular risk factors that were derived from the Framingham study and other epidemiologic studies (age, gender, BP, cholesterol, diabetes, and smoking) fall short of explaining cardiovascular morbidity in patients with CKD (29). Consequently, this has led to investigations into novel, putative risk factors that may explain this discrepancy.

SDMA: More than an Expensive Creatinine?

SDMA in our study was a good marker of eGFR by the Modification of Diet in Renal Disease formula. It is well established that renal function predicts cardiovascular events in different patient populations (29). One of the recent large, community-based studies by Go *et al.* (31) showed that a reduced eGFR correlates with the risk for death, cardiovascular events, and hospitalization. Therefore, our finding of the association between eGFR and coronary calcification score is not surprising. However, that in the multiple regression analysis only SDMA significantly correlated with the stenosis score suggests that it is either one of two things: SDMA either is the ultimate marker of renal function's being more sensitive than all other known markers to estimate GFR and thereby able to pick up even small changes in GFR or, alternatively, might be an independent marker or even a mediator for atherosclerosis, above and beyond renal function. Surprising, ADMA did not correlate with the stenosis score, although it is regarded as a risk factor for CVD (12), and SDMA was superior in predicting GFR and the stenosis score. This is in line with a recent study by Fliser *et al.* (18), who suggested both that ADMA and SDMA can be a marker of progression of renal disease and poor cardiovascular outcome. Also in patients with idiopathic arterial pulmonary hypertension, generation of SDMA was markedly increased (32). Valkonen *et al.* (33) could show that SDMA is elevated in patients with previous coronary events as compared with control subjects without previous coronary heart disease. Future larger studies have to test whether SDMA is a convenient and clinically reliable marker of GFR that is independent of interindividual variability as a result of muscle mass, protein intake, age, and gender. A further advantage of SDMA may be its sensitivity in very early stages of renal disease.

Why Did SDMA not Predict Cardiovascular Events and Mortality in Previous Studies?

The biggest study that examined methylarginines ADMA and SDMA and mortality is the one by Zoccali *et al.* (11) in patients with ESRD, in which SDMA indeed did not predict cardiovascular outcome. This comes as no surprise, however, because both creatinine and SDMA rise exponentially with decreasing renal function (CKD stage 5), resulting in marked changes of these parameters caused by minute changes in GFR. In our study, however, patients with CKD stage 5 were excluded. Other studies on mortality and dimethylarginines in nonrenal patients (9) or patients with CKD 1 through 4 (34) failed to quantify SDMA. A recent study by Wanby *et al.* (35), involving 363 patients, reported that there was a significant positive multivariate relationship between SDMA and cardioembolic infarction.

What Could Be the Underlying Mechanism of SDMA as a Marker of Cardiovascular Risk?

Besides its good correlation with renal function, we know little about the effect of SDMA on the L-arginine–NO pathway. Closs *et al.* (22) showed that SDMA had no effect on the inducible NOS that was extracted from macrophages but competed for L-arginine transport. Tojo *et al.* (23) reported that SDMA reduced significantly the L-arginine uptake from the perfused loop of Henle of the rat kidney. This evidence suggests that SDMA is a potent competitor of L-arginine transport and thereby still could have an indirect inhibitory effect of NO synthesis by limiting arginine availability to NOS. Furthermore, the arginine transporter and eNOS are physically associated in the caveolae of endothelial cells (36) that provide a mechanism for the directed delivery of extracellular arginine to eNOS. This supports the finding that NO synthesis is sensitive to changes in extracellular arginine availability (37). Moreover, a number of *in vivo* and *in vitro* studies indicated that NO production by vascular endothelial cells can be increased by supplemental L-arginine (38,39). Finally, there is evidence that the reduced bioavailability of L-arginine results in uncoupled eNOS (40). Under these conditions, eNOS generates superoxide anion to increase oxidative stress, attenuate NO bioactivity, and induce endothelial dysfunction.

Results from this study are consistent with these observations. We found that SDMA dose-dependently reduced NO synthesis and increased ROS formation. This finding is supported by the observation that co-incubation with the NOS substrate L-arginine but not its D-enantiomer completely inhibited the effect of SDMA on NO synthesis and ROS formation, confirming the involvement of SDMA to reduce L-arginine availability to NOS.

SDMA is synthesized by PRMT 7 and PRMT 5 (both type II methyltransferases), which also produce small amounts of ADMA (13,14). It was shown recently that PRMT 5 regulates IL-2 gene expression (14), which indicates a link between SDMA elaboration and inflammation. In this study, we found a significant correlation of SDMA with IL-6 ($r^2 = 0.383$, $P < 0.0001$) and for ADMA ($r^2 = 0.192$, $P < 0.018$). As Tripepi *et al.* (41) showed, serum IL-6 added the highest prediction power to a cardiovascular death model in patients with ESRD, C-reactive protein may be considered as a cheap alternative. In our study, SDMA was not significantly correlated with C-reactive protein, which does not exclude, however, a relationship between PRMT II activity (and SDMA generation) and inflammation.

Although this study is limited by its design and the sample size, our data suggest the benefit of measuring and reporting SDMA beyond estimation of renal function. For further elucidating the difference in biologic activity between ADMA and SDMA and especially for studying the role of SDMA in CVD, the use of analytical assays that differentiate ADMA and SDMA with high specificity (*e.g.*, HPLC, LC-MS) is necessary. Measurement of SDMA could be done easily with a cheap, fast, and rugged method, if a LC-MS is available (42), which is the case in most routine laboratories.

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