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Dimethylarginines at the crossroad of insulin resistance and atherosclerosis

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Abstract

We tested if asymmetric dimethylarginine (ADMA) contributes to the simultaneous evolution of atherosclerosis and insulin resistance. We investigated the significant predictors of insulin resistance in the context of atherosclerosis, focusing on the role ADMA, symmetric dimethylarginine (SDMA), and L-arginine play in a cohort of young atherosclerotic patients and their age-matched controls. In a case-control study, 60 patients younger than 55 years having at least 30% stenosis of the internal carotid artery and 30 age- and sex-matched controls were recruited at a community-based neurosonologic laboratory. We found a strong positive association between the homeostasis model assessment of beta-cell function and insulin resistance and the ADMA/SDMA ratio that remained statistically significant even after adjusting for all significant and a priori identified determinants (β = 6.76; 95% confidence interval [CI], 2.13-11.39; P = .005). Interestingly, this relationship was even more pronounced in the atherosclerotic stratum (β = 8.29; 95% CI, 1.43-15.15; P = .019), whereas, on multiple linear regression, lack of association was seen in subjects free of carotid atherosclerosis (β = 1.39; 95% CI, −5.46 to 8.26; P = .671). We conclude that ADMA/SDMA ratio is a significant determinant of insulin resistance and may be a better parameter to monitor than ADMA alone. By accounting for the competition at the y+ transporters, ADMA/SDMA ratio could be an indicator of intracellular ADMA level.

1. Introduction

Evidences in support of asymmetric dimethylarginine (ADMA), the most significant endogenous nitric oxide synthase (NOS) inhibitor, being a cardiovascular risk molecule are constantly accumulating [1]. As the details of the mechanisms accompanying this effect are unraveling, attention is starting to divert toward the symmetrical stereoisomer of ADMA—symmetric dimethylarginine (SDMA) [2]. Symmetric dimethylarginine, although it lacks a direct inhibitory effect on NOS, may hinder the synthesis of nitric oxide by competing with both ADMA and L-arginine for cell entry via the concentrative y+ transporter. Albeit some studies failed to associate SDMA with all-cause mortality and fatal/nonfatal cardiovascular events [3,4], more sophisticated approaches focusing on SDMA have identified risks associated with its elevation [5,6]. Accordingly, in the multicenter Coronary Artery Risk Determination investigating the Influence of ADMA Concentration (CARDIAC) study, SDMA contributed to risk stratification in patients whose ADMA level was below the threshold level (1.75 μmol/L) of increased risk for coronary heart disease [7].

Earlier, on the basis of clinical evidences and preclinical models using NOS inhibitors for inducing insulin resistance, our group has proposed that ADMA may contribute to the simultaneous evolution of atherosclerosis and insulin resistance by inhibiting the endothelial and the neuronal NOS of the anterior hepatic plexus, with the latter being associated with the regulation of insulin sensitivity [8].

Starting from this hypothesis, we set out to investigate the significant predictors of insulin resistance in the context of atherosclerosis, with special focus on the role ADMA, SDMA, and L-arginine play in a cohort of young atherosclerotic patients and their age-matched controls.

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2. Materials and methods

2.1. Study design and protocol

All clinical investigations described in this study have been conducted in accordance with the guidelines set forth in the Declaration of Helsinki and were approved by the Ethical Committee of the University of Debrecen (Debrecen, Hungary). Informed consent was obtained from each participant.

Young (upper age limit of 55 years) atherosclerotic patients (cases) and their age-and sex-matched controls were recruited at the neurosonologic laboratory of the Department of Neurology, University of Debrecen. The presence of atherosclerosis was established on the basis of the status of the internal carotid artery (ICA); patients having at least 30% stenosis upon duplex ultrasound examination were designated into the atherosclerotic group. The main outcome measure of interest was insulin resistance defined by the homeostasis model assessment (HOMA) of beta-cell function and insulin resistance. Insulin-resistant state was defined as a HOMA index of higher than 4.4 [9].

Blood samples were drawn in the morning between 7:30 and 8:00 AM after an overnight fast, and before the administration of the morning medications. Serum samples were frozen within 60 minutes of being drawn and were stored at −70°C until analysis.

Serum glucose was determined in duplicate using the glucose oxidase method (Beckman, Fullerton, CA), whereas other laboratory parameters were determined using standard laboratory procedures as described elsewhere [10]. Insulin was quantified using the Insulin-ct (MP Biomedicals, Solon, OH) kit according to the manufacturer's instructions.

Immediately after blood sampling, ultrasound examinations were performed with a color-coded HP SONOS 2000 (Hewlett Packard, Riverside, CA) carotid duplex equipped with a 7.5-MHz linear transducer. For screening, the ICA stenosis was classified into categories of 10%, taking into account peak systolic velocity in the jet of the stenosis, the broadening of the stenotic and poststenotic spectra, peak systolic velocity in the poststenotic ICA, and direction of ophthalmic flow. A peak systolic velocity of at least 120 cm/s was the threshold for a 50% stenosis [11].

2.2. Determination of ADMA and other arginine derivatives

The solid-phase extractions were achieved adapting the method of Nonaka et al [12]. Serum of 250 μL was mixed with 50 μL L-homoarginine hydrochloride (Sigma, Steinheim, Germany) as internal standard (1000 μmol/L) and 700 μL borate buffer (pH 9.00), then the solutions were passed through the solid-phase extraction cartridges (OASIS MCX 3cc) using a 12-column manifold (J. T. Baker, Philipsburg, NJ). After the washing procedure, the arginine derivatives were eluted with a solution of (in milliliters) ammonia-water-methanol (10/40/50, vol/vol/vol) (using ammonia solution from Reanal [Budapest, Hungary] and methanol from Scharlau [Sentmenat-Barcelona, Spain]). The solvent was evaporated to dryness at 60°C in vacuum then dissolved in 200 μL deionized water and used for derivatization as described by Molnar-Perl and Vasanits [13]. The samples of 200 μL were mixed with 63 μL *ortho*-phthaldialdehyde/3-mercaptopropionic acid reagent solution. Subsequently, the samples were incubated at 22°C for 10 minutes then were cooled down to 5°C. For chromatography, samples of 20 μL were injected into the chromatographic system consisting of a Waters 2695 Separations Module equipped with thermostable autosampler (5°C) and column module (35°C) and of a Waters 2745 Fluorescent detector with a Waters Symmetry C-18 (4.6 × 150 mm, 3.5 μmol/L) column (Waters, Milford, MA). Gradient elution at a flow rate of 1 mL/min was applied using mobile phase A (20 mmol/L $(NH_4)_2CO_3$ in acetonitrile [Scharlau] and water with a ratio of 10:90, pH adjusted 7.50 ± 0.05) and mobile phase B (acetonitrile). The gradient condition was as follows: 0 to 13 minutes at 100% A; 13 to 15 minutes linear change to 70% A and 30% B, with the setting held for additional 5 minutes (ie, 15-20 minutes); 20 to 20.1 minutes linear change to 100% A, held until 25 minutes. Analytes were detected at λ_{ex} = 337 nm; λ_{em} = 20 nm was used for arginine and homoarginine, and λ_{em} = 454 nm, for ADMA and SDMA. Baseline separation was obtained.

The intraday precision for arginine, ADMA, and SDMA were 2.2%, 2.1%, and 1.2%, respectively. Recoveries were better than 98% for each component, and the limit of detection and quantification were 0.10, 0.05, and 0.05 μmol/L, respectively.

2.3. Statistical analysis

Normality of continuous variables was checked by the Shapiro-Wilk test. In case of normal distribution, Student *t* test was used for comparison. Frequencies were compared by the Pearson χ^2 test. Indicator coding was used in case of missing variables. Linear regression was used to characterize the relationship between insulin resistance and ADMA or SDMA level. An exploratory linear regression procedure was performed to obtain the best multiple model identifying the factors linked to HOMA index and estimating the effects thereof, expressed as coefficients. Accordingly, simple linear regression was performed with possible determinants of insulin resistance (defined as elevated HOMA index), including serum creatinine level; traditional risk factors (age, sex, triglyceride, cholesterol, high-density lipoprotein cholesterol [HDL-C], low-density lipoprotein cholesterol, apolipoprotein (Apo) A-I, apo B, lipoprotein(a), glucose, insulin, history of diabetes, heart disease and cerebrovascular disease, hypertension [defined as blood pressure >140/90 mm Hg], smoking habit); emerging risk factors (C-reactive protein, fibrinogen, homocysteine); serum ADMA, SDMA, and L-arginine; and the ratios of ADMA to SDMA and L-arginine to ADMA. The initial multiple model was based on variables identified a priori on the basis of critical evaluation of available scientific literature and as the result of simple modeling (the absolute value of the coefficient >0 at significance <.05). Accordingly, the following variables were included: ratio of ADMA to SDMA,

age, sex, arterial hypertension, smoking, diabetes, and serum creatinine (identified in advance), as well as the significant determinants serum triglyceride, HDL-C, apo A-I, and history of cerebrovascular disease. Variables were entered in the model simultaneously, and thereafter, insignificant determinants not contributing to the model were deleted one by one. The final model contained all variables identified a priori, as well as HDL-C, triglyceride, and history of cerebrovascular disease. Finally, to assess the possible interaction between atherosclerosis and insulin resistance, we performed the analysis of the data set stratified according to the status of the ICA (non-atherosclerotic vs atherosclerotic). Values are given as means or regression coefficients and their 95% confidence intervals. Statistical analysis was performed by Stata 8.2 (Stata Corporation) software.

3. Results

The baseline demographic characteristics and laboratory values are summarized in Table 1. The mean age of all subjects was 50.7 years, with insulin-resistant patients being slightly but statistically significantly younger than their insulin-sensitive counterparts (49.73 vs 51.09 years). Men and women were distributed equally between groups, and there were no differences in the prevalence of carotid atherosclerosis, diabetes, heart disease, hypertension, smoking, cardiovascular diseases, and antiplatelet use. Cerebrovascular disease was more frequent in the case histories of insulin-resistant patients than in those of controls (Table 1).

The mean serum ADMA levels were 0.40 ± 0.01 μmol/L (95% CI [confidence interval], 0.39-0.42), 0.41 ± 0.09 μmol/L (95% CI, 0.39-0.43), and 0.39 ± 0.09 μmol/L (95% CI, 0.36-0.43) in the complete data set, the insulin-sensitive stratum, and the insulin-resistant stratum, respectively. Similarly, L-arginine level did not differ significantly with respect to insulin sensitivity, whereas serum SDMA level showed pronounced reduction among insulin-resistant individuals (0.37 ± 0.12 μmol/L [95% CI, 0.34-0.39] and 0.31 ± 0.09 μmol/L [95% CI, 0.27-0.35] for the insulin-sensitive and insulin-resistant stratums, respectively). Conversely, the ratios of L-arginine to ADMA and ADMA to SDMA were unaltered and significantly increased, respectively. Serum creatinine level was not statistically different between insulin-sensitive and insulin-resistant patients (Table 1).

Conversely, we found a negative association between SDMA and insulin resistance but not between ADMA and

Table 1
Characteristics and risk factor profiles in insulin-sensitive and insulin-resistant individuals

Feature	Insulin sensitive (n = 64)	Insulin resistant (n = 26)	P
Age (y)	51.09 ± 3.39	49.73 ± 3.63	.047
Sex (male/female)	37/27	14/12	.73
ADMA (μmol/L)	0.41 ± 0.09	0.39 ± 0.09	.25
SDMA (μmol/L)	0.37 ± 0.12	0.31 ± 0.09	.017
Arginine (μmol/L)	88.17 ± 14.54	89.22 ± 16.21	.38
ADMA/SDMA	1.15 ± 0.22	1.33 ± 0.31	.017
Arginine/ADMA	223.37 ± 47.49	232.74 ± 49.3	.80
Components of the metabolic syndrome			
Triglyceride (mmol/L)	1.68 ± 1.14	2.49 ± 1.62	.004
HDL-C (mmol/L) in men	1.41 ± 0.46	1.09 ± 0.27	.016
HDL-C (mmol/L) in women	1.44 ± 0.39	1.15 ± 0.32	.009
Hypertension (Y/N)	35/28	20/6	.059
Fasting glucose (mmol/L)	4.35 ± 0.46	5.6 ± 1.82	<.001
Other factors			
Creatinine (μmol/L)	94.44 ± 102.76	76.16 ± 13.04	.44
Insulin (μIU/mL)	14.71 ± 3.65	39.78 ± 23.51	<.001
Cholesterol (mmol/L)	5.61 ± 1.12	5.52 ± 0.76	.35
LDL-C (mmol/L)	3.48 ± 1.03	3.36 ± 0.84	.31
Apo A-I (g/L)	1.6 ± 0.33	1.42 ± 0.25	.007
Apo B (g/L)	1.11 ± 0.32	1.15 ± 0.25	.26
Lp(a) (mg/L)	355.82 ± 494.2	302.1 ± 342.77	.3
CRP (mg/L)	5.8 ± 20.03	6.42 ± 5.39	.44
Homocysteine (μmol/L)	13.47 ± 5.17	12.5 ± 4.61	.2
Carotid atherosclerosis (Y/N)	40/24	20/6	.19
Diabetes (Y/N)	3/61	3/23	.25
Cerebrovascular disease (Y/N)	18/46	13/13	.048
Heart disease (Y/N)	14/50	10/16	.11
Antiplatelet use (Y/N)	35/29	16/10	.8
Smoking (Y/N)	31/33	11/15	.6

Values represent cell frequencies or mean ± SD. *P* value denotes the statistical significance of *t* test for normally distributed continuous variables and Pearson χ^2 test for frequencies. Creatinine values were not distributed normally; thus, Kruskal-Wallis test was used for comparison. Lp(a) indicates lipoprotein(a); CRP, C-reactive protein.

Table 2
Determinants of the HOMA index (insulin resistance) on the basis of simple linear regression

Parameter	Coeff	CI		P
		Lower limit	Upper limit	
ADMA (100 nmol/L)	−0.043	−1.30	1.21	.95
SDMA (100 nmol/L)	−0.91	−1.86	0.047	.062
Arginine (μmol/L)	0.002	−0.07	0.075	.95
ADMA/SDMA	7.44	3.49	11.4	<.001
Arginine/ADMA	−0.007	−0.23	0.22	.95
Homocysteine (μmol/L)	0.026	−0.19	0.24	.81
Creatinine (μmol/L)	−0.004	−0.019	0.012	.625
Triglyceride (mmol/L)	1.17	0.39	1.95	.004
Cholesterol (mmol/L)	−0.29	−1.36	0.78	.59
LDL-C (mmol/L)	−0.025	−0.13	0.076	.62
HDL-C (mmol/L)	−3.99	−6.47	−1.51	.002
Apo A-I (g/L)	−3.82	−7.20	−0.45	.027
Apo B (g/L)	1.02	−2.63	4.68	.58
Lp(a) (mg/L)	−0.0003	−0.003	0.002	.80
CRP (mg/L)	0.02	−0.045	0.083	.56
Insulin (μIU/mL)	0.28	0.26	0.3	<.001
Glucose (mmol/L)	2.66	1.92	3.39	<.001
Age (y)	−0.033	−0.35	0.28	.83
Sex	−0.37	−2.56	1.82	.74
Carotid atherosclerosis	1.33	−0.96	3.62	.25
Diabetes	2.85	−1.49	7.19	.20
Cerebrovascular disease	3.34	1.16	5.52	.003
Heart disease	1.99	−0.43	4.42	.105
Hypertension	2.35	0.15	4.56	.037
Smoking	−1.63	−3.78	0.52	.14

Asymmetric dimethylarginine and SDMA are given in units of 100 nmol/L. Coeff indicates regression coefficient.

insulin resistance (β = −0.055; 95% CI, −0.106 to −0.044; P = .033 and β = 0.014; 95% CI, −0.055 to 0.026; P = .489, respectively).

Abnormalities of the lipid homeostasis were more prevalent in the insulin-resistant group, with triglyceride level being significantly higher, whereas apo A-I and HDL-C were significantly lower among insulin-resistant individuals.

The result of simple linear regression used to identify the significant predictors of insulin sensitivity (HOMA index) is shown in Table 2. The strong positive association seen between the HOMA index and the ADMA/SDMA ratio remained statistically significant even after adjusting for all significant predictors and a priori identified confounders (β = 6.76; 95% CI, 2.13-11.39; P = .005). Interestingly, this relationship was even more pronounced in the atherosclerotic stratum (β = 8.29; 95% CI, 1.43-15.15; P = .019), whereas upon multiple linear regression, a lack of association was seen in subjects free of carotid atherosclerosis (β = 1.39; 95% CI, −5.46 to 8.26; P = .671).

4. Discussion

The major finding of the present study is that the ratio of ADMA to SDMA is positively correlated with HOMA index, a commonly used measure of insulin sensitivity and beta-cell function in early-onset atherosclerosis. In addition, we found an inverse relationship between these 2 parameters in patients free of carotid atherosclerosis. That this significant positive correlation is limited only to the atherosclerotic stratum supports the hypothesis that dimethylated arginine derivatives are indeed at the intercept of the processes contributing to insulin resistance and atherosclerosis. In addition, consistent with the National Cholesterol Education Adult Treatment Panel III criteria for metabolic syndrome, we found that the insulin-resistant patients had elevated triglyceride and fasting glucose levels and diminished HDL-C level [14]. Moreover, apo A-I levels were also reduced among our insulin-resistant patients (for review of lipid changes in insulin resistance, see reference [15]).

Upon reviewing our data, we found that the change in the ADMA/SDMA ratio comes from the significant decrease of SDMA in insulin resistance, with the ADMA level being practically unaltered. In accordance with this, we found that insulin resistance was negatively correlated with SDMA but not ADMA levels.

Several mechanisms may contribute to the decrease of SDMA levels in insulin resistance, for example, increased cellular uptake or enhanced elimination. It has been previously reported that hyperinsulinemia is able to induce the expression of y+ transporters that serve as the primary route for cellular uptake of L-arginine, ADMA, and SDMA [16,17]. In fact, any 1 of these 3 substrates competitively inhibits the entry of the 2 other congeners to a point that even SDMA devoid of direct NOS inhibitory action is able to limit the cellular nitric oxide synthesis by blocking the uptake of L-arginine [2]. Nevertheless, to the best of our knowledge, there are no available data providing details concerning the affinity or the selectivity of these transporters toward ADMA and SDMA. Our data are in agreement with this concept as shown by serum insulin level being negatively correlated with SDMA but not ADMA or L-arginine (data not shown). Yet another mechanism that may contribute to the decrease of SDMA is the enhanced renal elimination that arises in response to the hyperfiltration seen in the prediabetic and diabetic states [18,19]; however, the fact that renal function characterized by serum creatinine level fails to differ between the 2 groups makes this possibility rather unlikely. Finally, Siroen et al [20] have recently shown that the liver is able to clear both ADMA and SDMA from the systemic circulation, thus, the paradigm that SDMA is exclusively eliminated by renal mechanism does not seem to stand any longer. The observation of Ogawa et al [21] supports these findings. In their previous work, they demonstrated the presence of alternative routes for dimethylarginine metabolism, which yield α-ketoacid analogues. Starting from the fact that the liver is the organ of central importance in glucose homeostasis, it may be proposed that the hepatic metabolic pathways of SDMA are somehow up-regulated in insulin resistance. One possible series of events may be as follows: ADMA catabolism is decreased in insulin resistance syndrome owing to oxidant stress. This, however, is compensated by hepatic ADMA catabolism. Hepatic SDMA catabolism is likewise up-regulated, resulting in decreased SDMA level.

The lack of association between ADMA and insulin resistance (depicted by HOMA index) contradicts the observations of Stuhlinger and colleagues [22] and McLaughlin et al [23] who, in their landmark article, reported a strong positive correlation between plasma ADMA concentration and insulin resistance (characterized by the steady-state plasma glucose concentration) independent of other typical factors known to accompany the risk cluster termed *metabolic syndrome*. However, there are other reports that, similar to ours, also reported the lack of a significant difference between ADMA levels when patients with normal and abnormal coronary endothelial function were compared, with a median HOMA index of 9.6 and 13.8 (P = .004), respectively [24]. It may be argued that the HOMA index is not a pure measure of insulin resistance as it accounts for the alteration of beta-cell function as well; however, it is accepted as a validated model for the characterization of insulin resistance for the purposes of epidemiologic studies [9,25,26].

Previously, we proposed that ADMA may cause insulin resistance by the inhibition of the neuronal isoform of NOS (nNOS), whereas the simultaneously observed atherosclerosis is the consequence of endothelial NOS inhibition [8]. This hypothesis stemmed from animal models using intraportal administration of nonselective and selective nNOS inhibitors for inducing insulin resistance by hindering a potent insulin-sensitizing mechanism referred to as

meal-induced sensitization, anatomically linked to the nitrergic fibers of the anterior hepatic plexus [27]. In addition, various preclinical and clinical studies have demonstrated a role for ADMA in atherogenesis [28-32].

Starting from fact that the 2 stereoisomers ADMA and SDMA compete with each other for entry into the cell via the y+ transporter, we propose that their ratio is a better measure of ADMA entering the cell than ADMA alone. Consequently, the increase in the ratio of ADMA to SDMA could be viewed as a relative increase of ADMA. More specifically, the decrease of SDMA in the presence of unaltered ADMA level may contribute to the evolution of both insulin resistance and atherosclerosis by failing to oppose to ADMA at the site of entry (the y+ transporters), thereby facilitating ADMA's cellular uptake and the subsequent endothelial NOS/nNOS inhibition. Our finding that the ratio of ADMA to SDMA is a significant determinant of insulin resistance in atherosclerosis further articulates the possibility that ADMA assumes a central role in the evolution of both of these disease entities [8].

One possible shortcoming of the study design is the lack of data concerning body weight, waist circumference, or body mass index, factors closely interconnected with insulin resistance, as metabolic syndrome is often defined with the aid of these parameters. Given the fact, however, that our aim was not the investigation of the possible determinants of the metabolic syndrome, rather, that of beta-cell dysfunction and whole-body insulin resistance, we feel that the present work is an adequate exploration of this problem.

In summary, we found that the ADMA/SDMA ratio was significantly correlated with the HOMA index in patients affected by early-onset atherosclerosis. Accordingly, we propose that the ratio of ADMA to SDMA, by accounting for the competition at the y+ transporters, may be an indicator of intracellular ADMA level; thus, it may be a significant factor in determining insulin sensitivity.

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