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Plasma levels of asymmetric dimethylarginine in patients with biopsy-proven nonalcoholic fatty liver disease

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Abstract

Asymmetric (ADMA) and symmetric dimethylarginine (SDMA) are produced by breakdown of proteins that have been methylated posttranslationally at an arginine residue. Plasma levels of ADMA are elevated in insulin resistance states. Nonalcoholic fatty liver disease (NAFLD) is associated with insulin resistance and varying degrees of hepatic dysfunction. Because ADMA is metabolized in the liver, we hypothesized that ADMA levels will be high in patients with NAFLD as a consequence of hepatic dysfunction and insulin resistance. Plasma levels of ADMA, SDMA, total homocysteine, glucose, and insulin were measured in nondiabetic patients with biopsy-proven NAFLD (11 steatosis and 24 nonalcoholic steatohepatitis) and 25 healthy subjects. Plasma ADMA levels were significantly higher (P = .029) in patients with biopsy-proven NAFLD (0.43 ± 0.21 μmol/L) compared with controls (0.34 ± 0.10 μmol/L). However, when adjusted for insulin resistance (homeostasis model assessment), the difference between 2 groups was not evident. Plasma SDMA levels were similar in all 3 groups. Plasma levels of ADMA were positively correlated with plasma total homocysteine levels (P = .003). Plasma levels of SDMA were negatively correlated with estimated glomerular filtration rate (P = .016) and positively correlated with plasma total homocysteine levels (P = .003). The ratio of ADMA/SDMA was positively correlated with body mass index (P = .027). Elevated plasma concentrations of ADMA in biopsy-proven NAFLD were primarily related to insulin resistance. Hepatic dysfunction in NAFLD does not appear to make significant contribution to changes in plasma methylarginine levels.

1. Introduction

Methylated arginines $N^{G'}$-methyl-L-arginine (monomethylarginine, MMA), N^G,$N^{G'}$-dimethyl-L-arginine (symmetric dimethylarginine, SDMA), and N^G,N^G-dimethyl-L-arginine (asymmetric dimethylarginine, ADMA) are generated by the cleavage of proteins that are posttranslationally methylated at the arginine residues. Proteins with methylated arginines play essential regulatory role, including signal transduction, RNA transcription, and DNA repair [1]. Asymmetric dimethylarginine and MMA are inhibitors of nitric oxide synthase that catalyzes the conversion of L-arginine to nitric oxide, a potent endogenous vasodilator [2,3]. Elevated levels of ADMA have also been identified as a risk factor for endothelial dysfunction [4,5]. Plasma levels of ADMA are related to its release from protein breakdown and to its disposal through its cleavage to dimethylamine and citrulline by the enzyme dimethylarginine hydrolase (DDAH). Although DDAH is present in the liver, pancreas, spleen, and kidney, hepatic DDAH plays a dominant role in the removal of plasma ADMA [6,7]. High circulating levels of ADMA are associated with hyperhomocysteinemia, a key intermediate in methionine metabolism. Studies of cardiac microvascular endothelial cells in culture showed that high levels of homocysteine inhibited the expression of DDAH and resulted in accumulation of ADMA in the medium [8,9]. Symmetric dimethylarginine is not metabolized by DDAH and is primarily eliminated by renal excretion; consequently, impaired kidney function results in higher levels of SDMA in the plasma [10].

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Higher plasma ADMA levels have been reported in insulin resistance states [11] and in subjects with type 1 and type 2 diabetes mellitus [12,13]. Plasma ADMA levels have been shown to decrease in response to improvement in insulin sensitivity in obese women [14]. In addition, hepatic dysfunction is associated with high plasma levels of ADMA [6,7,15]. Nonalcoholic fatty liver disease (NAFLD) is the hepatic component of metabolic syndrome and is associated with systemic insulin resistance [16]. Nonalcoholic fatty liver disease spans from steatosis, the accumulation of fat droplets in hepatocytes or fatty liver, to the more severe form, that is, steatohepatitis, characterized by steatosis plus lobular inflammation accompanied by ballooning degeneration of hepatocytes with and without fibrosis. Nonalcoholic fatty liver disease is associated with systemic insulin resistance and evidence of increased oxidative stress [16,17]. These metabolic perturbations have been suggested to impair hepatic DDAH activity and could result in changes in plasma concentrations of methylarginines [15,18]. In the present study, we have quantified plasma levels of ADMA and SDMA in nondiabetic subjects with biopsy-proven NAFLD and examined their relationship to insulin resistance, hepatic steatosis, and nonalcoholic steatohepatitis (NASH). We hypothesized that as a consequence of hepatic dysfunction, plasma ADMA levels would be higher in patients with NASH and steatosis as compared with healthy control subjects.

2. Methods

Thirty-five nondiabetic subjects with histologically diagnosed NAFLD (11 hepatic steatosis and 24 NASH) were recruited from the liver clinics of the Cleveland Clinic and MetroHealth Medical Center in Cleveland, OH. Liver biopsies were reviewed in a blinded manner by the same pathologist and given a NASH activity score (0-8) [19]. Subjects with diabetes mellitus and subjects with the plasma creatinine higher than 1.5 mg/dL were excluded from the study. Twenty-five healthy subjects in the control group had normal blood chemistry and no evidence of steatosis by ultrasound examination [20]. Written informed consent was obtained from all subjects. The protocol was approved by the Institutional Review Boards of MetroHealth Medical Center and the Cleveland Clinic Foundation.

Subjects reported to the General Clinical Research Center at 7:00 AM following 12 hours of fasting. After a 30-minute rest period, 3 venous blood samples were obtained 5 minutes apart for the measurement of plasma glucose and insulin concentrations. Additional blood was collected into EDTA-containing tubes. Blood samples were centrifuged at 4°C, and plasma obtained was stored at −80°C.

Monomethylarginine, monoethylarginine (MEA), ADMA, and SDMA standards were purchased from Calbiochem (Darmstadt, Germany). L-Arginine was obtained from Pierce (Rockford, IL). *o*-Phthaldialdehyde was from Fluka (Bucks, Switzerland). All other chemicals were obtained from Fisher (Pittsburg, PA) and Sigma-Aldrich (St Louis, MO). Oasis MCX cation-exchange solid phase extraction cartridges (1 mL) were purchased from Waters (Milford, MA).

Plasma ADMA and SDMA were analyzed by high-performance liquid chromatography (HPLC) using a fluorescent detector as described by Teerlink and colleagues [21] with minor modifications. Analytes were separated isocratically with the mobile phase consisting of 25 mmol/L potassium phosphate buffer (pH 6.5) with 7% acetonitrile. Monoethylarginine was used as an internal standard. This nonphysiologic L-arginine derivative is a preferable internal standard than homoarginine, MMA, or monopropylarginine [21,22] because both homoarginine and MMA are present in small quantities in human plasma. Monopropylarginine is a nonphysiologic compound; however, its chromatographic properties are similar to those of ADMA and SDMA. Fifty microliters of 2.8 μmol/L MEA (internal standard) was added to 200 μL of the standard solutions or to the plasma samples. The final concentration of internal standard in plasma samples was 0.56 μmol/L. Methylarginines were separated by solid phase cation-exchange chromatography. Analytes were eluted with 1 mL of a mixture 30% ammonium hydroxide, 1 mol/L sodium hydroxide, water, and methanol (10/0.5/40/50); evaporated to dryness; and then reconstituted in 100 μL water. Fifty microliters of reconstituted eluent was derivatized with 50 μL of freshly prepared 7.5 mmol/L *o*-phthaldialdehyde + 11.5 mmol/L 3-mercaptopropionic acid solution in methanol/potassium borate buffer, pH 9.5. The standard curves were constructed based on the chromatographic peak area ratios of MMA/MEA, ADMA/MEA, and SDMA/MEA. Intercepts of calibration curves were not significantly different from zero (regression coefficient, 0.99). The *lower limits of quantification*, defined as the lowest point in the calibration curve with a signal to noise ratio equal to 10, were 4.71, 4.05, and 5.06 ng/mL for MMA, SDMA, and ADMA, respectively. Intra- and interassay variation coefficients for SDMA and ADMA were less than 2.5% and less than 4.0%.

Plasma glucose was measured using the glucose oxidase method (Beckman glucose analyzer, Beckman Instruments, Fullerton, CA), and plasma insulin levels were measured using a commercial enzyme-linked immunosorbent assay kit (Linco Research, St Charles, MO). Homeostasis model assessment (HOMA) was calculated as a measure of insulin resistance (http://www.dtu.ox.ac.uk/homa). The HOMA model calculates insulin resistance based on simultaneous measurements of plasma glucose and insulin in overnight-fasted subjects [23]. Plasma total homocysteine levels were measured by HPLC [24]. Serum alanine and aspartate aminotransferases (AST and ALT, respectively) were measured by standard methods in the clinical laboratory.

Glomerular filtration rate (eGFR) was estimated using Modification of Diet in Renal Disease formula [25].

Table 1
Clinical and biochemical characteristics of the study subjects

Factor	Controls (n = 25)	Steatosis (n = 11)	NASH (n = 24)	P value
Age (y)	42.0 (9.4)	43.5 (10.7)	43.6 (12.6)	NS
Male	7 (28)	6 (54.6)	8 (33.3)	NS
BMI (kg/m^2)	23.3 (2.7)	34.0 (4.0)*	34.8 (4.7)*	<.001
Triglycerides (mg/dL)	77.0 (64.0, 93.0)	159.0 (115.0, 174.0)*	160.0 (94.5, 216.5)*	.007
Glucose (mmol/L)	4.7 (4.4, 4.8)	5.0 (4.5, 5.3)	5.1 (4.7, 5.5)*	.015
Insulin (pmol/L)	50.0 (39.4, 64.4)	111.6 (95.8, 188.9)*	147.0 (113.3, 236.1)*	<.001
AST	22.0 (18.0, 27.0)	30.0 (19.0, 37.0)	47.0 (32.0, 76.5)[†]	<.001
ALT	16.0 (13.0, 22.0)	35.0 (21.0, 61.0)*	58.0 (46.0, 118.5)*[,†]	<.001
Total homocysteine (μmol/L)[a]	6.5 (5.7, 8.9)	7.4 (6.7, 9.0)	8.8 (7.9, 10.6)*	.006
eGFR	86.3 (79.7, 96.2)	81.4 (74.1, 84.1)	88.6 (73.3, 97.0)	NS

Statistics presented are mean (SD), median (Q25, Q75), or number (percentage).
[a] Measured for 20 controls and 11 steatosis and 23 NASH patients.
* Significantly different from controls.
[†] Significantly different from controls and steatosis group ($P < .017$).

2.1. Statistical analysis

The data were analyzed by Student *t* test for comparison of results between control and combined NAFLD groups (steatosis + NASH). $P < .05$ was considered statistically significant. Analysis of variance was used to assess differences in continuous variables such as plasma levels of ADMA and SDMA and the ADMA/SDMA ratio. When at least one group was significantly different from the others, pairwise comparisons were performed using the Bonferroni adjustment for multiple comparisons. If the distributional assumptions were not met, then Kruskal-Wallis tests and Dunn multiple comparison procedure were used to compare the groups. Spearman correlation coefficients were used to assess associations between plasma ADMA and SDMA, ADMA/SDMA ratio, insulin resistance, and homocysteine. Finally, analysis of covariance was used to assess differences in plasma ADMA and SDMA and ADMA/SDMA ratio between the 3 groups adjusting for insulin resistance (HOMA). SAS version 9.1 software (The SAS Institute, Cary, NC) was used to perform all analyses.

3. Results

The clinical and biochemical characteristics of the study subjects are summarized in Table 1. Subjects with NAFLD had significantly higher ($P < .001$) body mass index (BMI; weight in kilograms/height in square meters) compared with controls. Body mass index was not significantly different among patients with steatosis and with NASH. Serum AST and ALT were significantly higher ($P < .001$) in patients with NASH compared with patients with steatosis and healthy controls. Plasma triglyceride and insulin concentrations were significantly higher ($P = .007$ and $P < .015$, respectively) in all patients with NAFLD than in control subjects (Table 1). Plasma glucose concentrations were significantly higher in NASH than healthy controls. Total homocysteine concentration in the plasma was significantly higher in subjects with NASH as compared with the controls. It should be underscored that plasma levels of total homocysteine in our population, controls and NAFLD, were within the reference range (<10 μmol/L). Estimated glomerular filtration rate was not significantly different between groups.

The calculated HOMA scores (measure of insulin resistance) were significantly higher ($P < .001$) in patients with biopsy-proven NAFLD (Table 2). Plasma ADMA and SDMA levels were not significantly different between controls and patients with steatosis or NASH (Table 2). Because the steatosis group was small (n = 11), we combined the steatosis and NASH, all NAFLD, for further analysis. When steatosis and NASH groups were combined, plasma levels of ADMA were significantly higher ($P < .015$) in patients with NAFLD (0.43 ± 0.21 μmol/L) as compared with the control subjects (0.34 ± 0.10 μmol/L) (Fig. 1). The circulating levels of SDMA were similar in controls and subjects with NAFLD. Higher plasma levels of ADMA in

Table 2
Insulin resistance, plasma ADMA and SDMA, and ADMA/SDMA ratio

Factor	Controls (n = 25)	Steatosis (n = 11)	NASH (n = 24)	P value
Insulin resistance (HOMA)	0.9 (0.7, 1.2)	2.2 (1.8, 3.4)*	2.8 (2.1, 4.3)*	<.001
ADMA (μmol/L)	0.34 (0.1)	0.41 (0.23)	0.45 (0.21)	.13
SDMA (μmol/L)	0.25 (0.08)	0.28 (0.13)	0.28 (0.11)	.59
ADMA/SDMA	1.38 (0.23)	1.49 (0.35)	1.61 (0.41)	.053

Data are median (Q25, Q75) for HOMA and mean (SD) for others. *P* values correspond to Kruskal-Wallis tests for HOMA and analysis of variance for others.
* Significantly different from controls ($P < .02$).

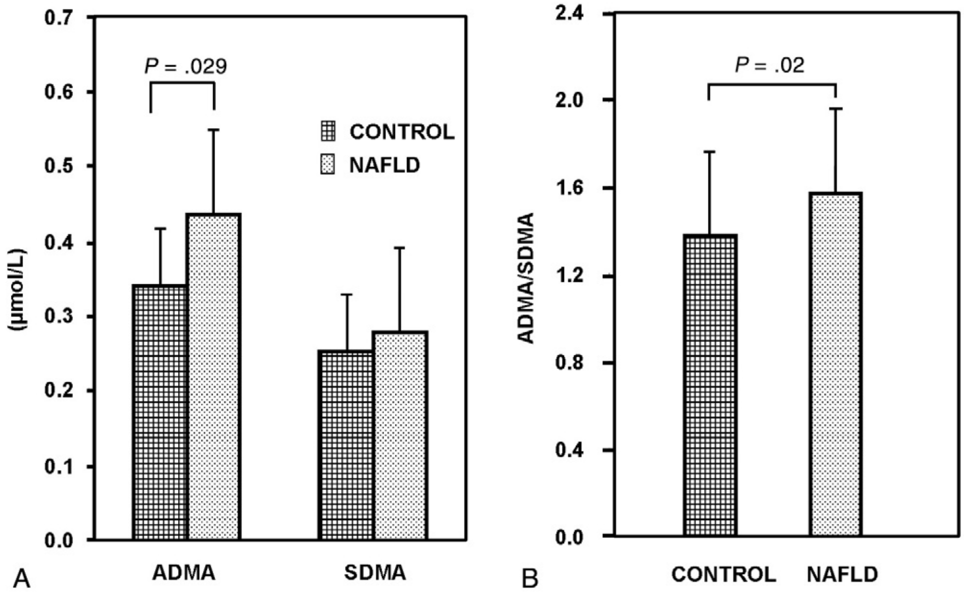

Fig. 1. The plasma concentrations of ADMA and SDMA (A) and the ADMA/SDMA ratio (B) in healthy controls and subjects with NAFLD.

patients with NAFLD resulted in significantly higher ($P < .02$) ADMA/SDMA ratio in these patients compared with that in controls (Fig. 1).

Because insulin resistance is accompanied by higher plasma levels of ADMA [11,14,24], we examined whether the increase in ADMA levels was related to insulin resistance or the liver disease. When adjusted for HOMA score, the plasma concentrations of ADMA and SDMA or the ADMA/SDMA ratios in patients with NAFLD were not significantly different from those in controls (Table 3).

We examined the relationship between ADMA, SDMA, ADMA/SDMA ratio, and clinical factors of interest in the entire subject population (controls and NAFLD, Table 4). As shown, plasma ADMA levels were positively correlated with plasma homocysteine levels ($\rho = 0.4$, $P = .003$) (Table 4). Plasma SDMA levels were negatively correlated with eGFR ($P < .02$). The ADMA/SDMA ratio was positively correlated with BMI ($\rho = 0.29$, $P = .027$) and with eGFR ($\rho = 0.3$, $P = .045$).

4. Discussion

In the present study, we observed that the plasma levels of ADMA and the ADMA/SDMA ratio, but not the plasma SDMA levels, were higher in nondiabetic subjects with biopsy-proven NAFLD compared with those in controls. There was no difference in ADMA levels between subjects with steatosis and those with NASH. The plasma level of ADMA correlated positively with plasma levels of total homocysteine in the entire population. The plasma concentrations of SDMA and the ADMA/SDMA ratio showed a significant correlation with the eGFR.

Plasma levels of ADMA in our control subjects were similar to those quantified by others using mass spectrometry [26–28] and HPLC techniques [21,22]. After adjusting for insulin resistance (HOMA), plasma levels of ADMA in patients with biopsy-proven NAFLD were not different from those in controls. This suggests that the insulin resistance is the primary contributor to the higher plasma concentration of ADMA in the NAFLD subjects. Other investigators have reported that higher plasma levels of ADMA are associated with insulin resistance [12,29]. The higher levels of ADMA in the insulin resistant state have been attributed to a higher rate of whole-body protein turnover [14,29]. Because plasma glucose concentrations were not significantly different among groups, the changes in ADMA may have been due to the differences in the plasma insulin concentrations.

Table 3
Plasma ADMA and SDMA and ADMA/SDMA values adjusted[a] for HOMA

Factor	Controls (n = 25)	Steatosis (n = 11)	NASH (n = 24)	*P* value
ADMA (μmol/L)	0.32 (0.23, 0.40)	0.42 (0.31, 0.53)	0.47 (0.38, 0.55)	.094
SDMA (μmol/L)	0.24 (0.19, 0.29)	0.28 (0.22, 0.35)	0.29 (0.24, 0.34)	.45
ADMA/SDMA	1.35 (1.18, 1.15)	1.50 (1.30, 1.70)	1.64 (1.48, 1.79)	.089

Values presented are mean (95% confidence interval).

[a] Adjusted means were obtained with an analysis of covariance analysis. Each factor (ADMA, SDMA, ADMA/SDMA) was modeled as the dependent variable, and independent variables were NAFLD group and insulin resistance.

Table 4
Correlations* between ADMA and SDMA and clinical factors of interest

Factor	ADMA		SDMA		ADMA/SDMA	
	ρ (95% CI)	P value	ρ (95% CI)	P value	ρ (95% CI)	P value
Insulin resistance (HOMA)	0.12 (−0.14,0.38)	.36	−0.01 (−0.27,0.25)	.94	0.23 (−0.03,0.48)	.079
Homocysteine	0.40 (0.14,0.65)	.003	0.40 (0.14,0.65)	.003	0.07 (−0.21,0.35)	.6
BMI	0.15 (−0.11,0.41)	.27	0.02 (−0.24,0.29)	.86	0.29 (0.03,0.54)	.027
eGFR	−0.08 (−0.36,0.21)	.59	−0.33 (−0.60,−0.07)	.016	0.28 (0.01,0.55)	.045
Albumin	−0.17 (−0.44,0.11)	.24	−0.18 (−0.45,0.10)	.21	0.20 (−0.07,0.48)	.14
Mean blood pressure	0.20 (−0.06,0.46)	.14	0.16 (−0.10,0.42)	.23	0.18 (−0.09,0.44)	.18

*Spearman correlation coefficients; P values correspond to testing the null hypothesis that $\rho = 0$ and deemed to be significant if $P < .05$.

Previous data show that hepatic dysfunction results in increased levels of ADMA in the plasma [7,15,30,31]. In critically ill patients, hepatic dysfunction was associated with elevated ADMA levels and was the strongest predictor of mortality [15]. Plasma ADMA concentrations were elevated in the hepatic vein of patients with compensated cirrhosis [31] and decreased following liver transplantation and recovery of liver function [32]. Our subjects were clinically compensated and did not show evidence of significant hepatic dysfunction. This may explain the lack of any observed differences in methylarginine concentrations between controls and NAFLD.

Increased generation of ADMA may also be related to an increased activity of protein methyltransferase (PRMT), the enzyme responsible for the methylation of arginine residue in cellular proteins. Cell culture studies in vitro have shown that inhibition of PRMT results in a reduction in ADMA synthesis by endothelial cells [4]. Increased hepatic expression of PRMT in patients with alcoholic hepatitis was accompanied by higher plasma ADMA levels [33]. Protein methyltransferase uses *S*-adenosyl-L-methionine as the methyl donor, resulting in the formation of *S*-adenosyl homocysteine and ultimately homocysteine. Although there is no direct evidence that high concentrations of homocysteine in vivo inhibit DDAH activity, in vitro data have demonstrated that homocysteine dose-dependently reduces the activity of recombinant human DDAH [34]. High homocysteine levels by inhibiting DDAH activity could result in an increase in plasma ADMA levels [2,33]. This is consistent with our observation of a significant positive correlation between plasma levels of ADMA and homocysteine levels (Table 4). A similar association between plasma levels of ADMA and homocysteine was also reported in a study of a general population [34].

The clinical significance of elevated plasma ADMA is related to its pathogenic role as an endogenous inhibitor of nitric oxide synthase and consequent endothelial dysfunction [3,6]. Patients with NASH have been reported to have significantly greater endothelial dysfunction compared with those with simple steatosis [6,34,35]. This may be a consequence of elevated plasma and tissue ADMA levels in these patients.

Our data show that the liver disease and insulin resistance in patients with NAFLD did not significantly affect the metabolism of SDMA. The positive correlation between plasma SDMA and eGFR is due to SDMA being excreted primarily by the kidney.

The small sample size of the steatosis group (n = 11) is a significant limitation of our present study. Thus, although there is a trend toward increase in methylarginine with disease severity (Table 2), the data are not statistically significant. Only a very large sample size (84 subjects in each group for 80%power) can show continuous change with severity of disease.

In summary, plasma ADMA and ADMA/SDMA ratio were higher in subjects with biopsy-proven NAFLD. Plasma levels of ADMA were also significantly correlated with the plasma concentration of total homocysteine. Compensated hepatic dysfunction did not appear to contribute to the elevated plasma levels of ADMA of patients with NASH.

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