Asymmetric dimethylarginine is closely associated with the development and progression of nephropathy in patients with type 2 diabetes

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Abstract

Background. Nitric oxide (NO) is thought to play an important role in the pathogenesis of diabetic nephropathy. We conducted a prospective, observational cohort study to explore the relationship between plasma levels of asymmetric dimethylarginine (ADMA), an endogenous inhibitor of NO synthase, and the development and progression of nephropathy in patients with type 2 diabetes.

Methods. This was a hospital-based observational cohort study in Japanese type 2 diabetic patients with normoalbuminuria [urinary albumin-to-creatinine ratio (ACR) <30 mg/g creatinine] or microalbuminuria (30 ≤ ACR <300 mg/g creatinine). The primary endpoint was the development or progression of diabetic nephropathy, based on transition from any given stage to a more advanced stage of albuminuria.

Results. We studied 225 diabetic patients, 81 women and 144 men, with a mean (±SD) age of 64 ± 10 years. The majority (183) of patients were normoalbuminuric, with the remainder microalbuminuric (42). During the median follow-up period of 5.2 years, 27 normoalbuminuric and 10 microalbuminuric patients reached the primary endpoint. When patients were separated according to the median ADMA level (0.46 μmol/l), patients with higher ADMA levels had a greater incidence of reaching the endpoint (P = 0.014 by the log-rank test). In the multivariate Cox proportional hazard model, the hazard ratio for reaching the endpoint for patients with higher versus lower ADMA levels was 2.72 (95% confidence interval 1.25–5.95; P = 0.012).

Conclusions. Higher plasma levels of ADMA may be a novel and potent predictor of the progression of nephropathy in adult Japanese type 2 diabetic patients.

Keywords: ADMA; diabetic nephropathy; GFR; microalbuminuria; nitric oxide

Introduction

Diabetic nephropathy is a major cause of end-stage renal disease worldwide [1]. Although the pathogenesis of diabetic nephropathy is unknown and likely multifactorial, recent studies suggest a potential link to decreased production of nitric oxide (NO) [2,3]. Decreased NO generation was observed in diabetic rat kidneys in vivo and in mesangial cell cultures exposed to high ambient glucose concentrations [4,5]. In a recent study involving diabetic mice lacking NO synthase, the progression of diabetic nephropathy was greatly accelerated, as shown by increased albuminuria and mesangial matrix expansion [6].

Asymmetric dimethylarginine (ADMA), the major endogenous inhibitor of NO synthase, is considered as a causative factor for endothelial dysfunction. Increased plasma levels of ADMA are associated with various clinical conditions involving endothelial dysfunction, including hypertension, hypercholesterolaemia, diabetes mellitus and cardiovascular disease [7–10]. In patients with non-diabetic kidney disease, circulating ADMA levels have been demonstrated to be positively correlated with the degree of proteinuria and a prognostic marker of progression of renal dysfunction [11,12]; however, it remains to be determined whether plasma ADMA levels are associated with diabetic nephropathy. We, therefore, conducted this observational cohort study to examine the association between plasma ADMA levels and the development and progression of nephropathy in patients with type 2 diabetes.

Subjects and methods

Study subjects

This was a hospital-based observational prospective cohort study. Japanese type 2 diabetic patients with normoalbuminuria or microalbuminuria were recruited from the outpatient clinic of the Diabetes Centre, Tokyo Women’s Medical University Hospital, Tokyo, Japan, during the period between April 2001 and July 2002. Type 2 diabetes was diagnosed according to the Japan Diabetes Association criteria [13].

At a regular ambulatory visit, subjects underwent an anthropometric and physical examination, including height, weight and blood pressure. Laboratory examinations included plasma glucose, haemoglobin A1C (A1C), serum lipids and creatinine using fasting blood samples, and urinary albumin measured in the first morning urine specimen. Patients with macroalbuminuria, defined as a urinary albumin-to-creatinine ratio (ACR) ≥ 300 mg/g Cr, were excluded. Patients who had a malignant disease, severe liver dysfunction or who had undergone lower limb amputation were also excluded.
The study protocol was designed in adherence to the Declaration of Helsinki, and informed consent was obtained from all subjects.

**Measurements**

Plasma ADMA levels were determined by high-performance liquid chromatography (HPLC). Urinary albumin was measured using the latex agglutination method and normalized by urinary creatinine. The stage of albuminuria at baseline was defined as normoalbuminuria (ACR <30 mg/g creatinine) or microalbuminuria (30 ≤ ACR < 300 mg/g creatinine) based on at least two out of three urinary ACR measurements according to the American Diabetes Association (ADA) guideline [14]. Glomerular filtration rate (GFR) was estimated using the following equation, originating from the Modification of Diet in Renal Disease (MDRD) Study group [15], and refitted for Japanese individuals: estimated GFR (eGFR) = 175 × SCr (mg/dl)^−1.154 × Age^−0.203 × 0.742 (if female) ÷ 0.741, where SCr = serum creatinine [16]. Serum creatinine was measured by Jaffe’s method and the value was calibrated, using the following equation, prior to inclusion in the equation: serum creatinine (enzymatic method in mg/dl) = 0.972 × serum creatinine (Jaffe’s method in mg/dl) – 0.224 (r = 0.999, P < 0.001). Triglycerides and total cholesterol were measured by enzymatic methods. A1C was measured by HPLC.

**Primary and secondary endpoints**

The primary endpoint of this study was defined as the transition from any given stage to a more advanced stage of albuminuria, which was established using at least two consecutive urinary ACR measurements to reduce misclassification. The secondary endpoint was the rate of change in eGFR. For each individual, the rate was determined by parameter estimates using a simple regression analysis, with eGFR as a function of time in years, applied to all eGFR values obtained during the follow-up period. Subjects were excluded from contributing to the secondary endpoint if they had <2.5 years of follow-up observation since the study entry. This minimum observation period was selected based on a previous recommendation for an observation period of at least 2 years for valid determination of the rate of decline in GFR [17].

**Statistical analysis**

Data were expressed as percentage, arithmetic mean ± standard deviation (SD) or geometric mean with 95% confidence interval (CI), as appropriate according to data distribution. For statistical analyses, the Student t-test, Fisher’s exact probability test, univariate and multivariate linear regression analyses and analysis of covariance (ANCOVA) were conducted according to the appropriate situation. In the univariate correlational analysis, Spearman’s correlation coefficients (rs) were calculated. The cumulative incidence of the transition of the albuminuria stage was estimated using the Kaplan–Meier method and the statistical difference between groups was compared by the log-rank test. Risk estimates for reaching the endpoint were calculated using univariate and multivariate Cox proportional hazard model analyses. The following covariates were used as conventional risk factors: age, sex, duration of diabetes, presence of simple and proliferative diabetic retinopathy, body mass index (BMI), systolic and diastolic blood pressure, usage of renin–angiotensin system (RAS) inhibitors (angiotensin-converting enzyme inhibitors and angiotensin receptor blockers), A1C, triglycerides, total cholesterol, eGFR and urinary ACR at baseline. P-values <0.05 were considered significant. In the multivariate linear regression analysis and the multivariate Cox proportional regression analysis, a stepwise variable-selecting procedure was performed, specifying the significant levels for entering another explanatory variable into the model as 0.25, and that for removing an explanatory variable from the model as 0.15, respectively. All statistical analyses were performed using the SAS version 9.13 (SAS Institute, Cary, NC, USA).

**Results**

**Baseline demographic and clinical characteristics**

We studied a total of 225 patients with type 2 diabetes, 81 women and 144 men, with a mean age of 64 ± 10 years. There were 60 patients treated with insulin, 107 patients treated with oral hypoglycaemic agents and 58 patients without any anti-diabetic medication. At baseline, 183 patients had normoalbuminuria, and the other 42 patients had microalbuminuria. The prevalence of retinopathy in microalbuminuric patients was significantly higher than that in normoalbuminuric patients (40.5% versus 75.0%, P < 0.001). The mean plasma ADMA level was 0.51 ± 0.15 μmol/l. Demographic and laboratory data in patients dichotomized by the median level of plasma ADMA (0.46 μmol/l) are presented in Table 1. Age, sex, BMI, blood pressure, presence of diabetic retinopathy, duration of diabetes, A1C, triglycerides, total cholesterol, creatinine, eGFR, medication for diabetes and usage of RAS inhibitors were comparable in the two groups.

**Cross-sectional association between plasma ADMA levels and albuminuria**

An association between plasma levels of ADMA and albuminuria at baseline was examined based on cross-sectional data. As shown in Table 1, patients with higher levels of ADMA had significantly increased urinary ACR compared to those with lower levels of ADMA. In the univariate correlational analysis, plasma ADMA levels were positively correlated with urinary ACR (rs = 0.21, P = 0.002) and negatively with eGFR (rs = −0.15, P = 0.028). Any other cardiovascular risk factors, HbA1c, systolic blood pressure, diastolic blood pressure, BMI, logarithmically transformed triglyceride or total cholesterol, have no correlation with plasma ADMA levels. To clarify independent effects, the multivariate regression analysis with a stepwise selection procedure was conducted including covariates listed in the Subjects and methods section. In this analysis, an increase in logarithmically transformed urinary ACR was selected as the only variable that was significantly associated with an increase in plasma ADMA levels (β = 0.064, P = 0.005), whereas eGFR was not selected in the model.

**Associations between plasma ADMA levels and the development and progression of albuminuria**

During the median follow-up period of 5.2 years (range: 0.3–6.4 years), 27 patients with normoalbuminuria and 10 patients with microalbuminuria progressed to a more advanced stage of albuminuria.

As shown in Figure 1, Kaplan–Meier estimates for time to reach the primary endpoint were significantly higher for patients with ADMA levels above than below the median level (log-rank test, P = 0.014). In the Cox proportional model analysis, the hazard ratio for the primary endpoint for patients with higher versus lower ADMA levels was 2.31 (95% CI 1.16–4.60; P = 0.017) in the univariate analysis and 2.72 (95% CI 1.25–5.95; P = 0.012) in the multivariate analysis adjusted for the conventional risk factors as indicated in the Subjects and methods section. When plasma ADMA levels at baseline were treated as a continuous variable, the hazard ratio for an increment of 0.1 μmol/l ADMA was 1.24 (95% CI: 1.03–1.51; P = 0.027) in the univariate analysis and 1.30 (95% CI: 1.04–1.63; P = 0.019) in
Table 1. Demographic and laboratory data in patients dichotomized by the median level (0.46 μmol/l) of plasma ADMA at baseline

<table>
<thead>
<tr>
<th>Variable at baseline</th>
<th>Below the median (N=111)</th>
<th>Above the median (N=114)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>63 ± 11</td>
<td>65 ± 9</td>
<td>0.120</td>
</tr>
<tr>
<td>Men (%)</td>
<td>76 (68.5)</td>
<td>68 (59.6)</td>
<td>0.211</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>23.3 ± 2.8</td>
<td>23.4 ± 3.1</td>
<td>0.821</td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>138 ± 18</td>
<td>138 ± 20</td>
<td>0.971</td>
</tr>
<tr>
<td>Diastolic blood pressure (mmHg)</td>
<td>76 ± 10</td>
<td>75 ± 11</td>
<td>0.486</td>
</tr>
<tr>
<td>Diabetic retinopathy, none/simple/proliferative (%)</td>
<td>58.9/30.0/11.1</td>
<td>46.6/40.8/12.6</td>
<td>0.224</td>
</tr>
<tr>
<td>Duration of diabetes (years)</td>
<td>13.2 ± 7.7</td>
<td>12.4 ± 7.4</td>
<td>0.396</td>
</tr>
<tr>
<td>Laboratory data</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Haemoglobin A1C (%)</td>
<td>7.1 ± 1.0</td>
<td>7.1 ± 1.0</td>
<td>0.835</td>
</tr>
<tr>
<td>Triglyceride (mmol/l)</td>
<td>1.16 (1.04–1.29)</td>
<td>1.16 (1.05–1.29)</td>
<td>0.953</td>
</tr>
<tr>
<td>Total cholesterol (mmol/l)</td>
<td>5.13 ± 0.78</td>
<td>5.10 ± 0.88</td>
<td>0.969</td>
</tr>
<tr>
<td>Creatinine (μmol/l)</td>
<td>84.0 ± 15.0</td>
<td>87.5 ± 20.3</td>
<td>0.190</td>
</tr>
<tr>
<td>Estimated GFR (ml/min/1.73 m²)</td>
<td>81.5 ± 19.2</td>
<td>76.6 ± 21.2</td>
<td>0.072</td>
</tr>
<tr>
<td>Urinary albumin (mg/g Cr)</td>
<td>9.4 (7.8–11.3)</td>
<td>13.0 (10.5–16.1)</td>
<td>0.026</td>
</tr>
<tr>
<td>Medication for diabetes, none/OHA/insulin (%)</td>
<td>29.7/49.6/20.7</td>
<td>21.9/45.6/32.5</td>
<td>0.108</td>
</tr>
<tr>
<td>RAS blockers (%)</td>
<td>24.3</td>
<td>36.0</td>
<td>0.061</td>
</tr>
</tbody>
</table>

GFR, glomerular filtration rate; OHA, oral hypoglycaemic agents; RAS, renin–angiotensin system.

Data are percent, mean ± SD or geometric mean (95% CI).

Categorical data were compared using Fisher’s exact probability test, and continuous data were compared by the Student t-test.

The multivariate analysis, both of which were statistically significant (Table 2).

Next, the Cox model analysis was conducted for the 183 normoalbuminuric and 42 microalbuminuric patients, separately. In the normoalbuminuric patients, univariate and multivariate hazard ratios for the primary endpoint for patients above the median level (0.46 μmol/l) were 2.07 (95% CI 0.95–4.53; P = 0.068) and 2.61 (95% CI 1.06–6.43; P = 0.037), respectively, relative to patients below the median. In the microalbuminuric patients, univariate and multivariate hazard ratios for patients above the median (0.51 μmol/l) were 5.55 (95% CI 1.17–26.25; P = 0.031) and 7.57 (95% CI 1.42–40.38; P = 0.018), respectively.

Table 2. Multivariate Cox proportional hazards regression analysis with a stepwise selection procedure to determine predictors of reaching the primary endpoint

<table>
<thead>
<tr>
<th>Variable at baseline</th>
<th>Adjusted hazard ratio (95% CI)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADMA (0.1 μmol/l)</td>
<td>1.30 (1.04–1.63)</td>
<td>0.019</td>
</tr>
<tr>
<td>Presence of proliferative diabetic retinopathy (yes versus no)</td>
<td>2.24 (0.93–5.41)</td>
<td>0.074</td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>1.03 (1.01–1.05)</td>
<td>0.002</td>
</tr>
<tr>
<td>Diastolic blood pressure (mmHg)</td>
<td>0.96 (0.93–1.00)</td>
<td>0.037</td>
</tr>
<tr>
<td>Log [Urinary ACR (mg/g Cr)]</td>
<td>1.89 (0.98–3.64)</td>
<td>0.057</td>
</tr>
</tbody>
</table>

ACR, albumin-to-creatinine ratio; ADMA, asymmetric dimethylarginine. Excluded variables from the model were age, sex, presence of simple diabetic retinopathy, duration of diabetes, body mass index, usage of renin–angiotensin system blockers, haemoglobin A1C, total cholesterol, logarithmically triglyceride and estimated GFR.

**Associations between plasma ADMA levels and the rate of change in eGFR**

The relationship between plasma levels of ADMA at baseline and the rate of change in eGFR was determined in 199 patients who were followed up for >2.5 years. The mean rate of changes in eGFR was significantly greater for patients with higher ADMA levels (−2.61 ± 2.96 ml/min/1.73 m²/year) than those with lower ADMA levels (−1.74 ± 1.93 ml/min/1.73 m²/year, P = 0.015 by the Student t-test). The significant difference between the two groups was retained after adjusting for covariates by ANCOVA (Figure 2).

**Discussion**

In this hospital-based prospective cohort study, we have demonstrated, for the first time, that in patients with type 2 diabetes, plasma levels of ADMA, an endogenous inhibitor of NO synthase, are associated with urinary albumin...
excretion in a cross-sectional analysis at baseline as well as with a faster progression of nephropathy, based on the transition to a more advanced stage of albuminuria and rate of change in eGFR. These relationships were confirmed by treating ADMA levels both as a continuous and categorical variable. Furthermore, these findings were independent of other variables that are well-known risk factors for the development of nephropathy in diabetic patients.

The cross-sectional results obtained in this study are in agreement with previous studies demonstrating a significant association between ADMA levels and proteinuria in non-diabetic patients [11,12]. These findings may suggest that albuminuria/proteinuria per se is a causative factor of elevated levels of ADMA in early diabetic kidney diseases. Alternatively, elevated ADMA levels may contribute to diabetic nephropathy and albuminuria. Indeed, Lejer et al. documented the predictive value of plasma ADMA levels for an increased rate of decline in GFR and incident end-stage renal disease in patients with type 1 diabetes [18].

One possible mechanism for increased ADMA levels in patients with diabetic nephropathy might be related to inactivation of dimethylaminohydrolase (DDAH), the enzyme that hydrolyzes ADMA in renal endothelial and tubular cells, due to glomerular proteinuria [12,19]. Another possible mechanism might be related to elevated plasma methylarginines, including ADMA, due to increased protein turnover, as proteinuria is known to increase protein turnover [20]. Furthermore, plasma ADMA is known to be cleared by the kidneys [11,21,22]; therefore, decreased GFR may also be responsible for the elevation of ADMA levels. However, a recent study has demonstrated that only a minor amount of ADMA is excreted by the kidneys in humans [23]. This is consistent with our results that no independent effects of eGFR were seen in the multiple regression analysis to predict ADMA levels.

Disruption of the NO synthase–NO system has recently been implicated as a causative factor for the pathogenesis of diabetic nephropathy [6,24]. Diabetic NO synthase knockout mice developed albuminuria and renal insufficiency faster than control diabetic mice [6]. Arteriolar hyalinosis, mesangial matrix expansion, mesangiosclerosis and Kimmelstiel–Wilson nodules were observed in these mice [6]. In addition, ADMA reduction by manipulation of DDAH significantly decreased proteinuria and inhibited the deterioration of GFR in rats with chronic kidney disease [24]. Interestingly, Sydow et al. recently documented that mice overexpressing DDAH-I had a 50% reduction in the insulin resistance index compared with wild type [25]. This may contribute to the association of ADMA with progression of nephropathy because insulin resistance has been demonstrated to be implicated in the pathogenesis of diabetic nephropathy [26]. Taken together, these observations suggest that ADMA may be a direct and indirect causative factor for the pathogenesis of diabetic nephropathy. Furthermore, elevated ADMA levels resulting from increased albuminuria might contribute to further progression of diabetic nephropathy in a vicious circle.

We found that, in both normo- and microalbuminuric diabetic patients, plasma ADMA levels had prognostic implications for the transition to a more advanced stage of albuminuria. It should be noted that these effects of ADMA were equal to or even stronger than those of well-known risk factors for the progression of diabetic nephropathy, including hyperglycaemia, hypertension and hyperlipidaemia in the Cox model. Measurement of the plasma ADMA level, therefore, may facilitate identification of diabetic patients at a greater risk for the development and progression of nephropathy in the clinical setting. Higher ADMA levels, associated with endothelial dysfunction, are known to be predictive of poorer outcomes in patients with cardiovascular diseases [10,18,27]. The predictive value of ADMA in diabetic patients with microalbuminuria needs to be further elucidated.

Our study has several limitations. First, we did not evaluate the time-dependent changes in plasma ADMA levels, glycaemia, blood pressure or lipidaemia during the follow-up period. In addition, plasma ADMA levels may be modified by certain medications, including RAS inhibitors [28–31]. Secondly, the original and refitted MDRD equations may have limitations in estimating GFR in individuals with normal or near normal kidney function [32,33]. Finally, this study was carried out in an urban university hospital in an ethnically homogenous population, which may not be representative of the entire type 2 diabetic patient population. Further studies will be needed to extrapolate these findings to the broader type 2 diabetic population.

In conclusion, this hospital-based observational prospective cohort study provides evidence that ADMA, an endogenous inhibitor of NO synthase, may be a predictor for the progression of nephropathy in adult Japanese patients with type 2 diabetes. Results of future trials on the effect of lowering plasma ADMA levels on the progression of diabetic nephropathy are eagerly awaited to formally test this hypothesis.

Conflict of interest statement. None declared.
References


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