Asymmetric dimethylarginine is negatively correlated with hyperglycemia in children

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Abstract. Asymmetric dimethylarginine (ADMA) is a nonselective nitric oxide (NO) synthase inhibitor associated with cardiovascular and metabolic disorders. In several prospective and cross-sectional studies, ADMA has evolved as a marker of cardiovascular risk. However, there is limited information on this serum marker in young people, particularly in those with obesity, type 1 diabetes (DM1) and type 2 diabetes (DM2). We investigated ADMA concentrations in children and adolescents with hyperglycemia as compared with healthy age- and sex-matched individuals. The subjects were 21 simple obesity [male 13, female 8; aged 11.7±4.3 years], 18 with DM1 [male 4, female 14; aged 12.9±4.2 years, duration of disease 3.4±2.1 years], 10 with DM2 [male 5, female 5; aged 13.9±3.4 years, duration of disease 2.8±1.4 years] and 21 controls [male 12, female 9; aged 11.1±2.7 years]. ADMA levels were analyzed in a cross-sectional study. Concentrations of serum ADMA were determined using an enzyme-linked immunosorbent assay. Circulating levels of ADMA were significantly lower in subjects with DM1, DM2 or obesity. In all subjects, ADMA levels were inversely correlated with glycated hemoglobin A1c concentrations ($r=-0.401$, $p=0.0003$) and serum glucose levels ($r=-0.341$, $p=0.0023$). Low circulating ADMA levels are directly associated with glucose levels, suggesting that ADMA production is suppressed in childhood in order to compensate and protect vasculopathy due to hyperglycemia.

Key words: Children, Diabetes, Glycosylated hemoglobin, Hyperglycemia

Asymmetric dimethylarginine (ADMA) is an endogenous nitric oxide synthase (NOS) inhibitor [1]. Circulating ADMA levels are correlated with cardiovascular risk factors such as hypercholesterolemia, arterial hypertension, diabetes mellitus, age and smoking [2, 3].

ADMA concentrations were directly correlated with plasma glucose concentrations in a population that included both diabetics and nondiabetics [4]. Plasma ADMA concentrations are reportedly elevated in adults with type 1 diabetes (DM1) [5, 6] and type 2 diabetes (DM2) [7]. A more pathogenic role for ADMA might be inferred from the positive relationship between long-term glycemic control and ADMA levels in DM2 [7]. However, this correlation has not yet been confirmed in DM1 [5, 8]. In addition, ADMA levels were reduced in adults with DM2 [9] and DM1 [10, 11], as well as in youths with DM1 [12-14] when compared with healthy control subjects, and negatively related to glycemic control [9]. This suggests a complex and variable effect of chronic hyperglycemia on plasma levels of ADMA.

Because the published data offer arguments for both higher and lower ADMA in patients with DM1 and DM2, the present study tested the undirected hypothesis that ADMA concentration differs significantly in pediatric patients with hyperglycemia (DM1 and DM2) without manifesting vascular complications, as well as healthy controls. We hypothesized that prolonged hyperglycemia may cause vascular abnormalities in nitric oxide (NO) action and ADMA-regulated NO biosynthesis.

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Methods

Subjects

Diabetic and obese subjects without macrovascular disease or microalbuminuria were recruited from October 2012 to January 2014 and attended the Kansai Medical University Pediatric outpatient clinic. This investigation was conducted according to the principles expressed in the Declaration of Helsinki. The study protocol was approved by Kansai Medical University Review Board for Human Studies (#1201), with written consent from the parents. Children were also asked to consent before entering the study. The study group consisted of 21 simple obese [male 13, female 8; aged 11.7±4.3 years, follow-up period 1.5±0.8 years], 18 with DM1 [male 4, female 14; aged 12.9±4.2 years, duration of disease 3.4±2.1 years], and 10 with DM2 [male 5, female 5; aged 13.9±3.4 years, duration of disease 2.8±1.4 years]. Diabetes was defined using the criteria of the American Diabetes Association [15]. Based on the body mass index (BMI) standard established by the Japanese Association for Human Auxology [16], obesity was defined as a BMI equal to or greater than the 90th percentile. None of the obese subjects were treated with medications, and did not show any evidence of endocrine malfunction or recent medication use. The control group consisted of 21 subjects [male 12, female 9, aged 11.1±2.7 years] with normal body weight, normal blood pressure and negative family history of diabetes mellitus.

All DM 1 subjects were insulin-treated (0.92±0.21 unit/Kg/day). All DM2 subjects were treated with metformin (250-750 mg/day). Absence of microalbuminuria was determined by measuring urinary albumin:creatinine ratio (<2.5 mg/mmol), and macrovascular disease was determined by the absence of history of a cardiovascular event.

Blood samples were collected in the morning. Blood cells were then separated by centrifugation and the resulting serum was stored at -80°C until analyzed. All samples used in the present study were stored under the same conditions.

ADMA and L-Arginine measurements

Serum ADMA levels were detected using the ADMA enzyme-linked immunosorbent assay (ELISA) kit (Immundiagnostik AG, Bensheim, Germany). The cross-reactivity was ≤0.5% for symmetric dimethylarginine (SDMA) and <0.02% for L-arginine. The lower limit of detection was 0.05 μmol/L. The inter- and intra-assay standard deviations were less than 0.03 % and 0.04 %, respectively. L-arginine levels were detected using the L-arginine ELISA kit (Immundiagnostik AG, Bensheim, Germany). The lower limit of detection was 3.0 μmol/L. The inter- and intra-assay coefficients of variation were less than 8.6 % and 8.5 %, respectively.

Serum nitrate/nitrite levels

The serum NOX [nitrite (NO2−)+nitrate (NO3−)] levels were measured using the colorimetric assay kit (Wako Pure Chemical Industries, Tokyo, Japan). Before measuring, samples were centrifuged through Micron YM-10 Centrifugal Filters (Micropore, Bedford, MA) to remove proteins from the serum.

Statistical analyses

Statistical analysis was performed using JMP 6 (SAS Institute Inc., Cary, NC) software. All values are given as the mean ± standard deviation (SD). Randomization checks using a 1-factor analysis of variance (ANOVA) were conducted for gender, age, BMI, glucose, HbA1c, uric acid, total cholesterol, HDL-cholesterol and creatinine (Table 1), as well as ADMA, NOx, L-Arginine and L-arg/ADMA ratio (Table 2). ANOVA analyses and pairwise comparisons with post hoc Tukey HSD were used to detect significance between groups. Regression line equations were calculated with the least-square method, and the differences between regression lines were tested using conventional regression comparison procedures. A 2-tailed P value of <0.05 was considered statistically significant in all analyses.

Results

The clinical characteristics of study subjects are listed in Table 1. All subjects with DM2 received metformin. All subjects with DM1 (n=18) were treated with only insulin. The mean plasma glucose concentrations were significantly higher in subjects with DM1 (p<0.01) and DM2 (p<0.05). The levels of glycated hemoglobin A1c (HbA1c) were significantly higher in DM1 and DM2 patients than in the control and obese subjects. Serum uric acid levels were significantly higher in obese and DM2 subjects than in control and DM1 subjects. Serum HDL-cholesterol concentrations were significantly lower in obese subjects than in the subjects of the other groups.
Low ADMA in children with hyperglycemia

In all subjects (Fig. 1 and Fig. 2). In addition, ADMA levels were inversely correlated with age (r=-0.352, p=0.0024). In a univariate correlation analysis, serum ADMA was not associated with serum uric acid, total-cholesterol, HDL-cholesterol or creatinine. No significant relationships were found between ADMA and insulin therapy dosage, gender or BMI.

Discussion

Our study has shown significantly lower levels of ADMA in subjects with uncomplicated DM1, DM2 and

Table 1: Study Population Characteristics

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Obese</th>
<th>Type 1 DM</th>
<th>Type 2 DM</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>21</td>
<td>21</td>
<td>18</td>
<td>10</td>
</tr>
<tr>
<td>Male/Female</td>
<td>12/9</td>
<td>13/8</td>
<td>4/14</td>
<td>5/5</td>
</tr>
<tr>
<td>Age (yr)</td>
<td>11.1±2.7</td>
<td>11.7±4.3</td>
<td>12.9±4.2</td>
<td>13.9±3.4</td>
</tr>
<tr>
<td>BMI (percentile)</td>
<td>43.6±21.9</td>
<td>97.5±3.1†</td>
<td>55.6±30.1</td>
<td>94.2±9.9†</td>
</tr>
<tr>
<td>Duration of diabetes (yr)</td>
<td>-</td>
<td>-</td>
<td>3.4±2.1</td>
<td>2.8±1.4</td>
</tr>
<tr>
<td>Glucose (mmol/L)</td>
<td>5.38±0.50</td>
<td>5.44±0.78</td>
<td>11.8±6.3†</td>
<td>8.16±2.39*</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>5.20±0.26</td>
<td>5.38±0.32</td>
<td>8.99±1.18†</td>
<td>6.75±1.07†</td>
</tr>
<tr>
<td>Uric Acid (mg/dL)</td>
<td>3.99±1.37</td>
<td>6.11±1.87†</td>
<td>3.73±1.10</td>
<td>6.13±1.87†</td>
</tr>
<tr>
<td>Total-Chol (mg/dL)</td>
<td>171±22</td>
<td>170±26</td>
<td>188±33</td>
<td>189±26</td>
</tr>
<tr>
<td>HDL-Chol (mg/dL)</td>
<td>59.8±14.7</td>
<td>42.6±11.7†</td>
<td>64.2±13.3</td>
<td>49.8±8.9</td>
</tr>
</tbody>
</table>

Values are mean±S.D. *p<0.05, †p<0.01 compared to controls.

Abbreviations: BMI, body mass index; HbA1c, glycated hemoglobin A1c; Total-Chol, Totalcholesterol; HDL-Chol, HDL cholesterol; yr, year

Table 2: Serum levels of ADMA, NOx, L-Arginine and Ratio L-arg/ADMA in the four study groups

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Obese</th>
<th>Type 1 DM</th>
<th>Type 2 DM</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADMA (μmol/L)</td>
<td>0.92±0.21</td>
<td>0.75±0.14†</td>
<td>0.67±0.15†</td>
<td>0.72±0.14†</td>
</tr>
<tr>
<td>NOx (μmol/L)</td>
<td>37.4±18.1</td>
<td>30.5±20.2</td>
<td>42.3±29.5</td>
<td>29.9±21.0</td>
</tr>
<tr>
<td>L-Arginine (μmol/L)</td>
<td>207±66</td>
<td>208±65</td>
<td>185±80</td>
<td>238±76</td>
</tr>
<tr>
<td>Ratio L-arg/ADMA</td>
<td>228±87</td>
<td>291±103</td>
<td>271±115</td>
<td>339±138*</td>
</tr>
</tbody>
</table>

Values are mean±S.D. *p<0.05, †p<0.01 compared to controls.

Abbreviations: ADMA, asymmetric dimethylarginine; NOx, nitrite (NO2^-)+nitrate (NO3^-); Ratio L-arg/ADMA, the ratio between L-arginine and ADMA

Fig. 1 Relationship between serum ADMA and plasma HbA1c levels in all subjects (r = -0.401, p=0.0003).

Fig. 2 Relationship between serum ADMA and plasma glucose levels in all subjects (r = -0.341, p=0.0023).

All study variables, such as ADMA, NOx, and L-arginine, were normally distributed. Serum ADMA levels were significantly lower in subjects with DM1, DM2 or obesity when compared to the controls (p<0.01, Table 2). Serum NOx and L-arginine concentrations were comparable among obese, DM1, DM2 and control subjects (Table 2). However, the L-arg/ADMA ratio was higher in DM2 subjects than in controls (p<0.05).

Univariate analysis revealed that ADMA levels were inversely correlated with HbA1c concentrations (r=-0.401, p=0.0003) and serum glucose levels (r=-0.341, p=0.0023) in all subjects (Fig. 1 and Fig. 2). In addition, ADMA levels were inversely correlated with age (r=-0.352, p=0.0024). In a univariate correlation analysis, serum ADMA was not associated with serum uric acid, total-cholesterol, HDL-cholesterol or creatinine. No significant relationships were found between ADMA and insulin therapy dosage, gender or BMI.
simple obesity compared to healthy control subjects. In addition, serum ADMA concentrations were inversely correlated with HbA1c and plasma glucose levels in all subjects. Serum ADMA concentrations did not correlate with total cholesterol, HDL-cholesterol, or other risk factors of atherosclerosis.

Our findings support the hypothesis that decreased NO inactivation by ADMA acts as an important mechanism for the impairment of endothelium-dependent relaxation in arteries exposed to high levels of glucose. The mechanisms by which high glucose levels decrease ADMA is not known. In this study, diabetic and obese subjects without macrovascular disease or microalbuminuria were recruited. Poor glycemic control may directly influence NO synthesis even in diabetic patients without renal complications, such as hyperfiltration or renal hyperperfusion [9]. Alternatively, hyperglycemia may lower ADMA by decreasing the production of ADMA or increasing the metabolism of ADMA. ADMA is predominantly cleared by the enzyme dimethylarginine-dimethylaminohydrolase (DDAH) in different tissues, such as liver and kidney [17]. It was previously reported that intensive insulin therapy modulates ADMA plasma concentration by preserving DDAH activity [18]. An acute reduction of ADMA plasma levels by insulin was demonstrated in young patients with DM1 [19]. These findings beg the question of whether glucose or insulin affects DDAH activity. In this study, insulin therapy did not correlate with serum ADMA concentration because ADMA levels were also decreased in the DM2 group without insulin therapy. Our findings suggest that glucose itself modifies ADMA metabolism.

Hyperglycemia is a primary cause of vascular complications in diabetes. A hallmark of these vascular complications is endothelial cell dysfunction, which is partly due to the reduced production of NO. Hyperglycemia and poor glycemic control may be the underlying mechanism of endothelial dysfunction in DM2 [20]. Hyperglycemia impairs DDAH and causes the accumulation of ADMA, which disturbs NO pathways in blood vessels [20]. On the other hand, prolonged exposure of endothelial cells to high glucose increases not only NO, but also superoxide anion production [21]. Chronic exposure to elevated glucose leads to an imbalance between NO and superoxide anion. The regulation of endothelial NOS (eNOS) activity is regulated through glucose-mediated mitochondrial production of reactive oxygen species [22]. The subjects with hyperglycemia showed decreased ADMA levels, which may be an indicator of impaired protection against oxidative stress. A negative correlation between ADMA and HbA1c, as seen in our study, was found in youth with DM1 [13, 23] and in adults with DM2 [9], but Abbasi et al. reported that plasma ADMA concentrations are 2-fold higher in untreated patients with DM2 compared with a matched control population [24]. Anderssohn et al. reported that the association between ADMA and DM per se remains controversial, and that such an association likely depended on the type and stage of diabetes [25]. The finding in our study of an inverse correlation between ADMA and age is consistent with a previous study [13]. Marcovecchio et al. reported that ADMA concentrations decreased with age, and suggested that chronic hyperglycemia might down-regulate mechanisms implicated in ADMA production or stimulate its metabolism, confounding short-term associations with complication risk [13]. Tamura et al. reported that urinary NOx and a biomarker of oxidative stress showed significant inverse correlations with age. Younger subjects grow rapidly and are likely exposed to high concentrations of reactive oxygen species and NO. Consequently, younger subjects are more vulnerable to the oxidation of DNA and biotissues [26]. Anticipated advances in clinical and experimental investigations will help us to better understand this complex interrelationship between age and ADMA.

We measured serum ADMA levels as well as L-arginine levels and found an increased ratio between L-arginine and ADMA (L-arg/ADMA ratio) in DM2 groups. Several studies support the view that the L-arg/ADMA ratio is important for the regulation of eNOS activity [27]. NOx is regulated by the concentrations of L-arginine and ADMA. The elevation in the L-arginine/ADMA ratio may explain the restored NO formation by endogenous L-arginine in DM2 patients [28]. In addition, DM2 subjects received metformin. Metformin, which is a structural analog of ADMA, has beneficial effects on the abnormalities associated with insulin resistance. Several papers reported that metformin lowers ADMA in patients with DM2 [29] or patients with polycystic ovary syndrome [30].

Some studies have reported increased ADMA levels in obese children [31], while others found no such correlation [32]. This discrepancy may be explained by the varying degrees of insulin resistance and hyperinsulinemia among patients. Obesity-induced endothelial dysfunction is associated with decreased NO pro-
duction, which is caused by impaired eNOS activity
and expression, and increased production of superox-
ide anion and ADMA as well as vasoconstrictor factors,
such as endothelin-1, and sympathetic nerve activation
[33]. Uslu and colleagues reported that ADMA levels
were correlated with hypoadiponectinemia and hyper-
leptinemia [34]. Adiponectin and leptin increases NO
production in vascular endothelium and inhibits endo-
thelial cell activation; both of these effects would be
impaired by decreased adiponectin levels, leptin resis-
tance and/or increased ADMA levels. Other factors,
with the exception of hyperglycemia, may indirectly
regulate ADMA metabolism.

A limitation of this study is the limited size of the
population sample, which may urge some caution when
interpreting our findings. In this study, we did not
investigate endothelial function as measured by bra-
chial artery flow-mediated dilatation, cardio ankle vas-
cular index, or pulse wave velocity as indicators of arte-
rial stiffness. Another limitation of our study is that we
did not measure protein arginine methyltransferases,
DDAH, or cationic amino acid transporters. Decreased
plasma ADMA levels could be caused by decreased
ADMA synthesis, increased ADMA metabolism,
increased transportation into tissues, or increased renal
excretion. Large-sized prospective studies on subjects
including children with healthy and metabolic syndrome
are warranted to further elucidate the factors affecting
ADMA levels.

In conclusion, paradoxically low circulating ADMA
levels are dependently associated with glucose levels,
suggesting compensatory down regulation of ADMA to
prevent cardiovascular disease in a pediatric population.

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**Author contributions**

J. Takaya and K. Kaneko designed the research, Y.
Tanabe and Y. Kuroyanagi conducted research. All
authors read and approved the final manuscript.

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