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The relationship between plasma asymmetrical dimethyl-L-arginine and inflammation and adhesion molecule levels in subjects with normal, impaired, and diabetic glucose tolerance

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Abstract

Increasing evidence suggests that the postprandial state is a contributing factor to the development of atherosclerosis. To evaluate the effects of acute hyperglycemia on endothelial dysfunction and inflammation, plasma asymmetrical dimethyl-L-arginine (ADMA), intercellular adhesion molecule 1 (sICAM-1), vascular cell adhesion molecule 1, and C-reactive protein (CRP) levels and secretory phospholipase A_2 ($sPLA_2$) activities were measured in subjects with normal (n = 35), impaired (IGT) (n = 25), and diabetic (DGT) (n = 20) glucose tolerance. At baseline, plasma ADMA, sICAM-1, and CRP concentrations and plasma $sPLA_2$ activities were higher in both the IGT and DGT groups than in the normal glucose tolerance group (for each comparison, each $P < .001$). Patients with DGT have higher plasma ADMA and sICAM-1 concentrations than patients with IGT (for each, $P < .001$).Two hours after glucose loading, plasma ADMA and CRP concentrations and $sPLA_2$ activities were significantly elevated in the 3 groups when compared with baseline levels (for each comparison, $P < .001$). Plasma vascular cell adhesion molecule 1 and sICAM-1 concentrations were found to be elevated from baseline levels after glucose loading in the IGT and DGT groups (for each comparison, $P < .001$). Correlation analysis at baseline suggested that there was a significant relationship between ADMA and inflammation and soluble adhesion markers in the studied groups. In conclusion, plasma concentrations of ADMA and of inflammation and adhesion molecules were elevated in the prediabetic state. A complex interrelation could exist between ADMA and inflammation, and mechanisms involved in endothelial dysfunction are multifactorial at the prediabetic and diabetic state.

1. Introduction

Endothelial dysfunction is involved in lesion formation by the promotion of both the early and late mechanisms of atherosclerosis including up-regulation of adhesion molecules, increased leukocyte adherence, and enhanced low-density lipoprotein (LDL) oxidation [1]. *Endothelial dysfunction* is a term that covers diminished production/availability of nitric oxide (NO) and/or an imbalance in the relative contribution of endothelium-derived relaxing and contracting factors [2]. It is now well established that major risk factors for cardiovascular diseases impact upon endothelial function by decreasing NO bioavailability. This condition may be caused by various mechanisms including decreased NO synthesis, increased NO degradation due to oxidative stress, or reduced sensitivity to NO [3]. Nitric oxide is synthesized from L-arginine by NO synthases. Recently, it has been suggested that the methylated L-arginine metabolite asymmetrical dimethyl-L-arginine (ADMA), which is a competitive NO synthase antagonist, likely acts as an autocrine regulator of endothelial NO synthase activity and not only is a biochemical marker of atherosclerosis but also is thought to play a causal role in its genesis [4]. Boger et al [5] indicated that ADMA may be a novel risk factor for endothelial dysfunction in humans.

It is widely accepted that inflammation is an integral feature of atherosclerosis [6]. Vascular inflammation occurs in response to injury induced by various stimuli, such as oxidative stress. This subject is supported by the recent clinical findings that C-reactive protein (CRP) is an independent risk factor for coronary heart disease and has been widely used as a clinical marker of the state of inflammation [7]. The other molecule that is involved in

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inflammation is secretory phospholipase A_2 ($sPLA_2$). Secretory phospholipase A_2 participates in lysophosphatidylcholine formation, which has a potent activity to induce endothelial dysfunction and lesion formation [8]. Secretory phospholipase A_2 increases LDL oxidation and promotes the formation of bioactive phospholipids via the release of polyunsaturated free fatty acids, resulting in an enhanced ability to stimulate monocyte-endothelium interaction [9].

During the activation of endothelial cell, the expression of adhesion molecules, such as intercellular adhesion molecule 1 (sICAM-1) and vascular cell adhesion molecule 1 (sVCAM-1), is also increased, leading to the rolling, activation, and adhesion of leukocytes to the endothelium [10]. Antibody-blocking studies and studies using genetically modified mice have implicated these functions of adhesion molecules in the regulation of leukocyte recruitment to sites of inflammation and ischemia-reperfusion injury, and in atherogenesis [11].

Impaired glucose tolerance (IGT) represents a state that increases the risk not only for type 2 diabetes mellitus but also for cardiovascular diseases. Endothelial dysfunction and low-grade inflammation are important abnormalities in people with IGT that may contribute to this dual risk [12]. The study of Deepa et al [13] shows that in Asian Indians, inflammatory markers (CRP, interleukin 6, and sVCAM-1) increase with increasing degrees of glucose intolerance. Plasma ADMA and its relationship with the plasma inflammatory markers are poorly reported in the prediabetic state. We investigated the plasma concentration of ADMA, CRP, sICAM-1, and sVCAM-1 and the plasma $sPLA_2$ activities at baseline and 2 hours after glucose loading.

2. Materials and methods

Subjects with normal glucose tolerance (NGT), IGT, and diabetic glucose tolerance (DGT) participated in the study. The state of glycemia was classified according to the American Diabetes Association criteria [14] after 75-g oral glucose tolerance test. Normal glucose tolerance was identified when fasting plasma glucose is <6.1 mmol/L and 2-hour postchallenge plasma glucose is <7.8 mmol/L (n = 35). Subjects with fasting plasma glucose <6.1 mmol/L and 2-hour postchallenge glucose levels between 7.8 and 11.1 mmol/L were identified as IGT (n = 25), and subjects with postchallenge plasma glucose >11.1 mmol/L were identified as DGT (n = 20). There were no specifics on evaluation of physical activity between groups. Exclusion criteria were any cardiovascular complications or inflammatory diseases, smoking, exercise, and any medications such as antioxidants, lipid-lowering drugs, or glucose-lowering drugs. All of the patients had normal blood pressure. Blood pressure analysis was obtained with the subjects in the sitting position after resting for 20 to 30 minutes. Measurements were performed on 3 different times. The average of 3 measurements was taken as the mean systolic and diastolic pressure.

All subjects showed no evidence of family history of diabetes. All subjects gave their informed consent before being tested. The study was approved by the ethics committee (no. 14797).

Because low carbohydrate intake may induce glucose intolerance, recipients were given meals including more than 150 g carbohydrate for 3 days (about 250 g/d) [15]. After a 12-hour fasting, baseline blood samples were collected; and subjects were challenged with the equivalent of 75 g anhydrous glucose dissolved in 250 mL water. No food or drink except water was allowed during the test. After 2 hours, postchallenge blood samples were collected. Venous blood samples were drawn into Li-heparin–containing tubes, avoiding venous stasis. One milliliter of whole blood was stored for glycated hemoglobin (HbA_{1c}) analysis, the remaining blood samples were centrifuged at 1500*g* for 10 minutes in a refrigerated centrifuge, and plasma was obtained. The samples were stored in −80°C until analysis.

Plasma ADMA concentrations were determined by competitive enzyme-linked immunosorbent assay (ELISA) assay (ADMA-ELISA; DLD, Hamburg, Germany). The ADMA in samples was acylated and competed with solid-phase bound ADMA for a fixed number of rabbit anti-ADMA antiplasma binding sites. The intraassay and interassay coefficients of variation were 3.4% and 4.5% for ADMA, respectively.

Plasma sensitive CRP concentrations were obtained by a method based on the principle of a solid-phase ELISA (hCRP ELISA; Biomerica, USA). The intraassay and interassay coefficients of variation were 3.2% and 3.5% for CRP, respectively.

Plasma $sPLA_2$ activity was measured using a specific substrate $sPLA_2$ that is converted into sulfhydryl products. Sulfhydryl products were detected colorimetrically using Ellman reagent 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB), which formed a yellow product with the sulfhydryl formed. The amount of $sPLA_2$ in the sample was compared with the amount of $sPLA_2$ in the standards by comparison of the yellow color generated ($sPLA_2$ enzyme assay; R&D Systems, USA, catalog no. DE2400). The intraassay and interassay coefficients of variation were 4.6% and 4.5%, respectively.

Plasma sVCAM-1 and sICAM-1-concentrations were assayed using a solid-phase sandwich ELISA (sVCAM-1 ELISA Kit; Diaclone Research [Tepnel], Besancon, France, and sICAM-1 ELISA Kit; Diaclone Research [Tepnel], respectively). A monoclonal antibody specific for sVCAM-1 and sICAM-1 was coated onto the wells of the microtiter strips provided. The intraassay and interassay coefficients of variation were 4.4% and 5.5% for sVCAM-1-1 and 4.7% and 5.2% for sICAM-1, respectively.

Plasma glucose, total cholesterol, triglyceride, high-density lipoprotein (HDL) cholesterol, urea, creatinine, total protein, and albumin levels were determined by enzymatic methods using commercial kits (Roche Diagnostics, Mannheim, Germany). The LDL cholesterol was calculated.

The HbA_{1c} determination was based on the turbidimetric inhibition immunoassay for hemolyzed whole blood (Roche Diagnostics). Plasma insulin levels were determined by solid-phase, 2-site chemiluminescent immunometric assay (Immulite; Euro/DPC).

2.1. Statistical analysis

Data are presented as the mean ± SD. For parametrically distributed data, comparisons between the groups were made using the paired *t* test and analysis of variance (ANOVA) followed by the Tukey honestly significant difference post hoc test. For nonparametrically distributed data, the Wilcoxon and Kruskal-Wallis tests were used where appropriate. Correlations between changes in variables were tested using the Pearson correlation. $P < .05$ was considered statistically significant.

3. Results

Demographic details and basic biochemical characteristics of the subjects with NGT, IGT, and DGT are given in Table 1. There were no significant differences between the groups in terms of age, body mass index, and blood pressure. Plasma total cholesterol, LDL cholesterol, HDL cholesterol, triglycerides, glucose, insulin, and blood HbA_{1c} did not differ significantly between the groups at baseline. As expected, 2-hour plasma glucose levels were significantly higher than baseline in all 3 groups ($P < .001$, $P < .001$, and $P < .001$).

At baseline, plasma ADMA, sICAM-1, and CRP concentrations and plasma $sPLA_2$ activities were significantly lower in the NGT group than in both the IGT ($P < .001$, $P < .001$, $P < .001$, and $P < .001$, respectively) and DGT groups ($P < .001$, $P < .001$, $P < .001$, and $P < .001$, respectively). No difference was found in plasma sVCAM-1 concentrations among groups. Plasma sICAM-1 concentrations were significantly higher in the DGT group than in the IGT group ($P < .001$) (Table 2). When compared with baseline levels, plasma ADMA and CRP concentrations and $sPLA_2$ activities were elevated in the NGT, IGT, and DGT groups (for each group, $P < .001$, $P < .001$, and $P < .001$, respectively); and plasma sVCAM-1 and sICAM-1 concentrations were elevated in the IGT and DGT groups (for each group, $P < .001$ and $P < .001$) 2 hours after glucose loading. There was a significant difference in both plasma ADMA and sICAM-1 levels between the IGT and DGT groups 2 hours after glucose loading ($P < .003$ and $P < .001$).

The results of the correlation analysis at baseline are as follows: There was a positive correlation between plasma ADMA concentrations and both plasma sICAM-1 ($P < .01$) and sVCAM-1 concentrations in the NGT group ($P < .01$). Plasma ADMA concentrations were correlated with plasma $sPLA_2$ activities in the NGT ($P < .01$), IGT ($P < .01$), and DGT groups ($P < .05$). In the IGT group, plasma ADMA concentrations were correlated with plasma sICAM-1 concentrations ($P < .01$). The relationship between CRP concentrations and plasma ADMA concentrations in the DGT group was found to be significant ($P < .05$) (Table 3). Plasma CRP concentrations were also related with both plasma $sPLA_2$ activities ($r = 0.475$, $P < .01$) and sVCAM-1 concentrations ($r = 0.490$, $P < .01$) in the DGT group. In the DGT group, plasma sICAM-1 concentrations were correlated with sVCAM-1 ($r = 0.400$, $P < .05$) and $sPLA_2$ activities ($r = 0.405$, $P < .05$).

4. Discussion

Subclinical inflammation is a risk factor for cardiovascular disease, and acute glycemia leads to oxidative and

Table 1
Demographic details and biochemical characteristics of the NGT, IGT, and DGT subjects (means ± SD)

	NGT subjects (n = 35)	IGT subjects (n = 25)	DGT subjects (n = 20)
Age (y)	47.3 ± 13.74	50.3 ± 10.8	47.7 ± 9.3
Body mass index (kg/m^2)	27.8 ± 5.3	27.1 ± 5.5	28.1 ± 5.4
Sex (female/male)	20/15	18/17	12/8
Systolic blood pressure (mm Hg)	95 ± 10	98 ± 9	99 ± 8
Diastolic blood pressure (mm Hg)	75 ± 5	77 ± 7	77 ± 5
HbA_{1c} (%)	5.24 ± 0.42	5.45 ± 0.34	5.78 ± 0.69
Urea (mmol/L)	5.0 ± 1.4	5.1 ± 0.9	6.4 ± 1.7
Creatinine (μmol/L)	72.3 ± 14.1	66.3 ± 8.8	69.8 ± 28.2
Total cholesterol (mmol/L)	5.12 ± 1.10	5.32 ± 1.03	5.38 ± 0.65
LDL cholesterol (mmol/L)	3.05 ± 0.82	3.21 ± 0.86	3.49 ± 0.71
HDL cholesterol (mmol/L)	1.43 ± 0.29	1.35 ± 0.35	1.22 ± 0.28
Triglycerides (mmol/L)	1.24 ± 0.40	1.25 ± 0.46	1.19 ± 0.52
Fasting glucose (mmol/L)	4.78 ± 0.85	5.07 ± 0.74	5.02 ± 0.51
2-h glucose (mmol/L)	5.50 ± 1.34 *	8.80 ± 0.98 *, †	12.13 ± 1.13 *, †, ‡
Fasting insulin (μU/mL)	9.8 ± 4.9	10.2 ± 4.9	11.2 ± 2.3

$P < .05$: statistical significance.
* $P < .001$ vs fasting glucose (paired *t test*).
† $P < .001$ vs NGT (ANOVA and Tukey post hoc test).
‡ $P < .001$ vs IGT (ANOVA and Tukey post hoc test).

Table 2
Plasma ADMA and inflammation and adhesion molecule levels in NGT, IGT, and DGT subjects (means ± SD)

Variables	NGT (n = 35)	IGT (n = 25)	DGT (n = 20)
ADMA (μmol/L)			
Baseline	0.30 ± 0.12	0.47 ± 0,13 †	0.54 ± 0.14 †
2 h after glucose loading	0.45 ± 0.18 *	0.81 ± 0.10*,†	0.98 ± 0.18*,‡,†
CRP (mg/L)			
Baseline	4.23 ± 1.91	7.78 ± 2.65 †	7.69 ± 2.52 †
2 h after glucose loading	5.50 ± 2.24 *	9.25 ± 2.70*,†	9.70 ± 2.56*,†
$sPLA_2$ (U/L)			
Baseline	447 ± 230	723 ± 274 †	756 ± 154 †
2 h after glucose loading	653 ± 173 *	1168 ± 244*,†	1260 ± 290*,†
sVCAM-1 (ng/mL)			
Baseline	1224 ± 255	1233 ± 267	1287 ± 220
2 h after glucose loading	1260 ± 294	1480 ± 297 †	1519 ± 250*,†
sICAM-1 (ng/mL)			
Baseline	434 ± 212	636 ± 129 †	867 ± 200†,§
2 h after glucose loading	433 ± 215	637 ± 175 †	1075 ± 184*,†,§

$P < .05$: statistical significance. Values are means ± SD.
* $P < .001$ vs baseline (P values obtained by paired t test).
† $P < .001$ vs NGT (P values obtained by ANOVA and Tukey post hoc test; for nonparametrically distributed data, by Kruskal-Wallis test).
‡ $P < .003$ vs IGT (P values obtained by ANOVA and Tukey post hoc test; for nonparametrically distributed data, by Kruskal-Wallis test).
§ $P < .001$ vs IGT (P values obtained by ANOVA and Tukey post hoc test; for nonparametrically distributed data, by Kruskal-Wallis test).

inflammation stress on endothelium [16]. The relationship between endothelial vasodilator dysfunction and inflammatory markers and their changes in response to glucose loading are not fully understood yet.

Asymmetrical dimethyl-L-arginine is a specific endogenous inhibitor of the NO synthase; and elevation of ADMA induces dysfunction of the endothelium, which becomes clinically evident by impaired endothelium-dependent vasodilatation, hyperaggregability of platelets, and enhanced monocyte adhesion [17,18]. In addition, it has been suggested that ADMA might be elevated under conditions of chronic hyperglycemia [19]. Stuhlinger et al [20] suggested that plasma ADMA concentrations were positively correlated with impairment of insulin-mediated glucose disposal in nondiabetics. In our study, baseline plasma ADMA concentrations were higher in the patients with IGT and DGT than in subjects with NGT. Although plasma ADMA concentrations were elevated after glucose loading in all groups, the ratio of increase in patients with DGT was significantly higher (81%) than that in subjects with both NGT (50%) and IGT (%70) when compared with those of baseline levels.

One of the possible links between ADMA formation and blood glucose might be explained on the basis of glucose-mediated oxidative stress and inflammation. In a previous study, we reported that NO availability was decreased when the blood glucose levels were only moderately elevated above normal levels; and it may be related with the enhanced oxidative stress [21]. In addition, ADMA is degraded by dimethylarginine dimethylaminohydrolase [4]. It has been suggested that a glucose-induced impairment of dimethylarginine dimethylaminohydrolase causes ADMA accumulation and may contribute to endothelial vasodilator dysfunction in diabetes mellitus [22].

C-reactive protein is a well-studied inflammatory marker in the setting of cardiovascular disease; and lastly, it has been suggested that increased CRP has also been associated with endothelial dysfunction and the progression of atherosclerosis [23]. In our study, plasma baseline CRP concentrations were increased at the prediabetic state, glucose loading caused an elevation in plasma CRP concentrations, and plasma CRP levels were found to be correlated with plasma ADMA levels at the prediabetic state. Although it has been shown that CRP can also promote endothelial dysfunction by quenching the production of NO and diminishing its bioactivity by increasing oxidative stress [24], the mechanisms are not completely clear.

Another novel inflammation marker is PLA_2. It has been postulated that, in an experimental model of diabetes, leukocyte activation was associated with augmented PLA_2 expression and that, in vitro, PLA_2 activity mediated leukocyte activation and inflammatory responses support the proatherogenic role for PLA_2 [25]. We found that plasma PLA_2 activities were increased after glucose loading. This might be the result of glucose-induced oxidative stress.

Table 3
Pearson correlation analysis (r) between plasma ADMA and inflammation and adhesion molecules in NGT, IGT, and DGT subjects at baseline

	NGT (n = 35) r	IGT(n = 25) r	DGT (n = 20) r
ADMA–sVCAM-1	0.470 *	NS	NS
ADMA–sICAM-1	0.485 †	0.425 *	NS
ADMA-sPLA2	0.455 †	0.450 †	0.400 *
ADMA-CRP	NS	NS	0.415 *

$P < .05$: statistical significance. NS indicates not significant.
* $P < .05$.
† $P < .01$.

Elevated PLA_2 activity was reported in response to oxidative stress [26], and its activity has been related with LDL oxidation [27]. Kita et al [28] reported that oxidized LDL serves an important role for the recruitment of inflammatory cells to the atherosclerotic vessel, as it up-regulates adhesion molecules on cells of the vessel wall. In a previous study, we found that LDL oxidation occurs at an early stage in diabetes [29]. On the basis of these findings, elevated $sPLA_2$ levels have been thought to be a link among elevated oxidative stress and inflammation. We found that plasma $sPLA_2$ activities were also correlated with plasma ADMA in all groups and were correlated with plasma sICAM in DGT group at baseline.

Adhesion molecules of immunoglobulin super family (sICAM-1 and sVCAM-1) are poorly expressed by the resting endothelium. In contrast, they are unregulated during atherogenesis [30]. Adhesion molecule levels in diabetes were reported to be elevated, unchanged, or related with hyperglycemia and/or insulin resistance [31-34]. Wautier et al [35] reported that glucose and glucose metabolites alter endothelial cell functions and induce adhesion molecule overexpression (sICAM-1, sVCAM-1). They suggested that hyperglycemia provoked an oxidant stress and that the formation of reactive oxygen intermediates perturbs NO formation and is deleterious for cell functions. Our findings indicate that only plasma sICAM-1 concentrations were high in the prediabetic state, but both plasma sICAM-1 and sVCAM-1 concentrations were increased with glucose loading. Plasma CRP levels were also found to be correlated with adhesion molecules. It has been reported that CRP can up-regulate sVCAM-1 and/or sICAM-1 expression [36]. Pasceri et al [37] found that CRP, at concentrations of 5 μg/mL, has significant proinflammatory effects in both umbilical vein and coronary artery endothelial cells. These findings support the hypothesis that CRP may play a direct role in promoting the inflammatory component of atherosclerosis. Therefore, we thought that increases in plasma CRP concentrations with elevated plasma ADMA levels may change the levels of adhesion molecules at the prediabetic state.

In conclusion, the increase in ADMA production that is related with increased inflammation might reduce NO bioavailability; and mechanisms involved in endothelial dysfunction are multifactorial at the prediabetic state. Although we showed a clear association between plasma ADMA levels and inflammation at the prediabetic state and short-term glucose loading might contribute to the ADMA accumulation, it remains unclear whether ADMA is causally related to changes in vascular function or perfusion after changes in inflammatory status and whether ADMA can serve as a marker of endothelial dysfunction in the presence of inflammation. Association between increased ADMA levels in plasma and inflammation marker may be an epiphenomenon of enhanced oxidative stress in IGT and DGT.

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