Serum Levels of Advanced Glycation End Products Are Associated With Left Ventricular Diastolic Function in Patients With Type 1 Diabetes

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OBJECTIVE — Impairment of left ventricular diastolic function, possibly caused by increased collagen cross-linking of the cardiac muscle, is common in patients with type 1 diabetes even without coronary artery disease. Advanced glycation end products (AGEs) cross-link tissue collagen and are found within myocardial fibers. The aim of this study was to examine for a possible association between circulating AGEs and left ventricular cardiac function.

RESEARCH DESIGN AND METHODS — Left ventricular diastolic and systolic function were assessed by M-mode and Doppler echocardiography in 52 patients with type 1 diabetes, age 40 ± 13 (mean ± SD) years, diabetes duration 17 ± 13 years, and HbA_{1c} 8.3 ± 1.1%. Serum levels of AGEs and N^{ϵ}-(carboxymethyl)lysine (CML) were measured by newly developed competitive immunoassays.

RESULTS — A positive correlation was found between serum levels of AGEs and isovolumetric relaxation time (IVRT), $r = 0.46$ ($P < 0.0008$), and left ventricular diameter during diastole, $r = 0.37$ ($P < 0.008$). The systolic parameters did not correlate with serum levels of AGEs. Stepwise regression analysis showed that 21% of the IVRT variation could be explained by serum levels of AGEs ($F = 11.4$, $P < 0.002$), whereas serum levels of CML, HbA_{1c}, albumin excretion rate, diabetes duration, and mean arterial blood pressure were of no importance. AGE levels were significantly increased in men compared with women ($P < 0.03$) and present or former smokers ($P < 0.04$).

CONCLUSIONS — Increased serum levels of AGEs, unlike serum levels of CML, are associated with heart stiffness in patients with type 1 diabetes, possibly mediated by the cross-linking properties of AGEs.

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Patients with type 1 diabetes are more prone to develop heart failure than patients without diabetes despite a comparable severity of coronary artery disease (1), possibly because of a specific diabetic cardiomyopathy (2). Cross-linking of collagen is thought to play a role in the development of diabetic cardiomyopathy (3). Nonenzymatic glycation of proteins or lipids can lead to the formation of reactive advanced glycation end products (AGEs), which are thought to be implicated in the formation of micro- and macrovascular complications (4–6) in diabetes. Although the AGE product N^{ϵ}-(carboxymethyl)lysine (CML) does not have the ability to cross-link collagen (7), most AGEs can increase the cross-linking of proteins, causing reduced tissue elasticity and decreased protein turnover (8). Early signs of reduced cardiac compliance include impairment of cardiac left ventricular diastolic function, which is a common entity in patients with type 1 diabetes even without signs of coronary artery disease (9–12).

We have recently developed immunoassays for AGEs and the glycoxidation product CML in serum (13,14), showing that serum levels of both AGEs and CML are increased in children with type 1 diabetes before they have developed microvascular complications (14,15). We have also shown that increased serum levels of AGEs can predict changes in kidney morphology in patients with type 1 diabetes and microalbuminuria (13). Because no studies have documented a connection between AGEs or CML and cardiac function in human diabetes, the primary objective of this study was to investigate whether there was an association between serum levels of AGEs and/or the non–cross-linking CML and diastolic and systolic cardiac function in patients with type 1 diabetes.

RESEARCH DESIGN AND METHODS

Subjects

Participating in the study were 52 type 1 diabetic patients drawn from a population of 74 consecutive patients attending the outpatient clinic of Frederiksberg Hospital, Denmark. There were 55 patients who were screened for diabetic complications (see below) and clinical signs of cardiovas-

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Abbreviations: AER, urinary albumin excretion rate; AGE, advanced glycation end product; BSA, bovine serum albumin; CML, N^{ϵ}-(carboxymethyl)lysine; CV, coefficient of variation; DT, deceleration time of the early flow velocity; IVRT, isovolumetric relaxation time; LVD, left ventricular diameter during diastole; LVEF, left ventricular ejection fraction; LVFS, left ventricular fractional shortening; LVS, left ventricular diameter during systole; peak A, peak velocity of late atrial filling; peak E, peak velocity of early filling; PWD, posterior wall thickness in diastole; R-R variation, beat-to-beat heart rate variation; SWD, septal wall thickness during diastole.

A table elsewhere in this issue shows conventional and Système International (SI) units and conversion factors for many substances.

cular disease, defined as missing peripheral pulses in the lower limb, amputations because of gangrene, and/or a verified history of ischemic heart disease or stroke. Blood was drawn to determinate the serum levels of AGEs and CML. M-mode and Doppler echocardiography was performed on resting subjects to measure echocardiographic parameters of left ventricular systolic and diastolic function. In 3 of these subjects, blood for AGE determination was lost, thus leaving 52 subjects in the study. Fundus ophthalmoscopies were performed by two trained ophthalmologists, who classified patients by the worse eye, according to the protocol for screening for diabetic retinopathy in Europe (16). The ophthalmologists were blinded to patient status. Overnight urinary albumin excretion rate (AER) was measured to classify patients as normoalbuminuric, microalbuminuric (two consecutive AERs 20–200 μg/min), or proteinuric (AER ≥200 μg/min). The actual AER at the time of the screening is given in Table 1. Autonomic neuropathy was evaluated in a separate test with the Valsalva maneuver and beat-to-beat variation during deep breathing six times per minute; <10 min^{-1} was considered to be an abnormal R-R (beat-to-beat heart rate variation) variation during deep breathing. Peripheral neuropathy was defined by bedside clinical criteria as normal, probable (diminished vibration sense or loss of one ankle jerk), or definite (loss of vibration sense and/or ankle jerks). Smoking status was obtained by interview.

Methods

AGE–bovine serum albumin, CML–bovine serum albumin, polyclonal anti-AGE-RNase antibodies, and monoclonal anti-CML antibodies. The AGE–bovine serum albumin (BSA) and polyclonal anti-AGE-RNase antibodies were produced by Makita et al. (17) and were identical to previously described anti-AGE antibodies (13). CML-BSA was prepared according to Reddy et al. (18). The monoclonal anti-CML antibodies (CML-2F8AxB) were identical to the ones recently described (14).

Delayed europium lanthanide fluorescence immunoassays for the quantification of AGEs and CML in serum. Serum levels of AGEs and CML were determined by previously published methods using competitive immunoassays with the DELFIA system (Wallac, Turku, Finland) (13,14). One AGE or CML unit was, according to Makita et al. (17), defined as the competitive activity of 1 μg of AGE-BSA or CML-BSA standard. The final serum concentrations of AGE or CML were corrected for total protein concentration. Serum samples were immediately frozen at −40°C until analysis (for up to 12 months). Data presented are means of triplicates. All samples were analyzed in one batch. The intra-assay coefficients of variation have been shown to be 8.7% (13) for the AGE assay and 6% for the CML assay (14).

M-mode and Doppler cardiography. Echocardiography was done after a 15-min rest and with the patients in the postprandial state, using a Vingmed 725 ultrasound machine (Vingmed A/S, Horten, Norway). 2-D guided M-mode echocardiography was carried out in the parasternal position according to the standardization of the American Society of Echocardiography (19). Pulse wave Doppler was obtained in the four-chamber apical view, positioned at the tips of the mitral leaflets (A and E measurements), and continuous wave Doppler was obtained in the apical long axis view, positioned through the left ventricular outflow tract and mitral valve (isovolumetric relaxation time [IVRT] measurements).

Blinded to other data of the patients, one of the cardiologists later measured left ventricular diameter during diastole and systole (LVD and LVS), together with septal and posterior wall thickness during diastole (SWD and PWD), from the videotapes. Left ventricular fractional shortening [LVFS = (LVD − LVS)/LVD] and ejection fraction [LVEF = $(LVD^3 - LVS^3)/LVD^3$] were calculated. Furthermore, left atrial diameter was measured. From the recorded flow velocity pattern, IVRT, peak velocity of early filling (peak E), deceleration time of the early flow velocity (DT), and peak velocity of late atrial filling (peak A) were measured. Intra- and interobserver variation of Doppler indexes of diastolic function are typically <10%, except for DT, which is 10–20% (20).

Other analyses. Glycated hemoglobin (HbA_{1c}) was determined with fast protein liquid chromatography (21), normal range 4.0–6.0% and coefficient of variation (CV) 3%, and AER was determined by immunoturbidimetry (22). Lipids were analyzed with enzymatic methods (Boehringer Mannheim, Mannheim, Germany). The total protein concentration in each serum sample was determined by the Biuret method (Boehringer Mannheim), which has a CV <2%.

Table 1—*Clinical characteristics of 52 patients with type 1 diabetes*

n	52
Age (years)	40.1 ± 12.5 (23–74)
Sex (M/F)	29/23
Duration of diabetes (years)	16.6 ± 13.1 (1–61)
HbA_{1c} (%)	8.3 ± 1.1
Mean 1-year HbA_{1c} (%)	8.2 ± 1.1
AER (μg/min)	8 (0–1,776)
Total cholesterol (mmol/l)	5.2 ± 1.1
HDL cholesterol (mmol/l)	1.66 ± 0.48
LDL cholesterol (mmol/l)	3.13 ± 0.92
Triglycerides (mmol/l)	0.95 ± 0.58
Systolic blood pressure (mmHg)	129 ± 19
Diastolic blood pressure (mmHg)	69 ± 11
Mean arterial blood pressure (mmHg)	92 ± 14
Heart rate (beats/min)	73 ± 11

Data are *n*, means ± SD (range), or medians (range).

Statistical analysis. Spearman's rank-order correlation was used to measure the strength of association between pairs of variables. Regression was analyzed with simple regression or with multiple linear regression with stepwise backward regression. The degree of explanation found by stepwise regression analysis was adjusted to take into account the number of independent variables, which reflected the degrees of freedom. Data are means ± SD unless stated otherwise. Comparisons between groups were analyzed by two-sided Mann-Whitney *U* test because data did not show a normal distribution. One-sided tests were used when analyzing serum AGE levels in smokers versus nonsmokers and patients with or without neuropathy, since the a priori hypothesis is that tobacco contains AGEs (23) and AGEs can cause microvascular complications. The level of significance was set at $P < 0.05$. Calculations were performed using Sigma Stat (Jandel, Erkrath, Germany).

RESULTS — Clinical characteristics are shown in Table 1, and the number of patients with micro- and macrovascular complications and data on smoking are shown in Table 2.

Table 2—*Micro- and macrovascular complications and smoking in 52 patients with type 1 diabetes*

Nephropathy	
Normoalbuminuria	37 (71)
Microalbuminuria	11 (21)
Proteinuria	4 (8)
Retinopathy	
Negative	25 (48)
Background	14 (27)
Maculopathy	3 (6)
Proliferative	10 (19)
Neuropathy	
Negative	40 (76)
Possible	6 (12)
Definite	6 (12)
Cardiovascular disease	
Antihypertensive treatment	13 (25)
Coronary heart disease	3 (6)
Verified stroke	1 (2)
Peripheral angiopathy	4 (8)
Smoking	
Never	20 (39)
Ex	9 (17)
Current	23 (44)

Data are *n* (%). A total of 15 patients had cardiovascular disease, and some had more than one specific cardiovascular disease.

Serum levels of AGEs and CML

The median (10–90 percentiles) serum levels of AGEs and CML were 10.2 (7.3–15.8) and 16.2 (11.3–25.2) U/ml, respectively. There was a significant correlation between serum levels of AGEs and serum levels of CML, $r = 0.55$, $P < 0.0001$. In a regression analysis using log AGE and log CML, CML could explain 27% of the variation in serum AGEs, log AGEs = 0.45 + (0.47 × log CML), $R^2 = 0.27$, $F = 17$, $P = 0.0001$.

Echocardiography

LVS and LVD and calculated LVFS and LVEF are indicated in Table 3. Mean values are within the normal range (24). In one subject with ischemic heart disease, ventricular function was slightly reduced (LVFS 23%). Otherwise, individual values were within the normal range. Wall thickness (SWD or PWD) was increased above the normal range (11 mm) (24) in nine subjects; four of these had hypertension.

Like the estimates of ventricular function, the average measures of diastolic function (Table 3) were within the normal range, mean ± 2 SD (25). Looking at the individual patients, however, revealed that 22 patients had a normal pattern and the rest had different abnormal patterns. Diastolic parameters were missing for one patient. A group of 16 patients had increased peak A, peak E, or both ($n = 8$); 8 of these were on antihypertensive treatment for microalbuminuria, hypertension, or both, comprising more than half of the total 13 subjects on antihypertensive treatment. A second group of nine had increased IVRT, either alone ($n = 3$) or in combination with changes in peak flow (partly overlapping with the first group). A third group of nine patients had either reduced peak E or increased DT. Subjects with clinical cardiovascular disease were found in all groups.

Association between serum levels of AGEs and CML and echocardiography

The serum levels of AGEs were positively correlated with IVRT, $r = 0.46$, $P < 0.0008$, and LVD, $r = 0.37$, $P < 0.008$, whereas serum levels of CML were positively correlated with IVRT, $r = 0.34$, $P < 0.02$, and the systolic variables LVFS, $r = 0.29$, $P < 0.04$, and LVEF, $r = 0.29$, $P < 0.04$. When excluding the patients with cardiovascular disease ($n = 15$), the positive correlation between AGEs and IVRT was still significant, $r = 0.52$, $P < 0.002$. Peak A was the only echocardiographic variable shown to be correlated with age, $r = 0.46$, $P < 0.001$. When adjusting for age in a multiple regression analysis, there were still no associations between serum levels of AGEs or CML and peak A.

In a stepwise regression analysis applying log IVRT, LVD, LVFS, and LVEF, these diastolic and systolic parameters were found to correlate with serum levels of AGEs or CML as dependent variables and with log AGE, log CML, log AER, HbA_{1c}, mean arterial pressure, and log diabetes duration as independent variables. Serum levels of AGEs could explain 21% of the variation in IVRT, $R^2 = 0.21$, $F = 11.4$, $P < 0.002$, whereas no variations in LVD, LVFS, and LVEF could be explained by variation in any of the independent variables.

Association between AGEs, CML, and clinical variables

The serum levels of AGEs and CML did not correlate with age, diabetes duration, HbA_{1c}, blood glucose, log AER, lipids, serum-creatinine, R-R interval variation to deep breathing or Valsalva maneuver, or blood pressure (data not shown).

Current or former smokers showed significantly increased levels of serum AGEs compared with those who never had smoked, 10.7 (7.8–16.5) U/ml and 9.5 (7.1–13.8) U/ml, respectively, $P < 0.04$. Men showed significantly higher serum AGE levels, 11.7 (7.5–15.7), than women, 9.0 (7.1–17.8), $P < 0.03$. Patients with possible/definite neuropathy also had higher serum CML levels: 10.9 (6.3–16.5) U/ml vs. 10.2 (7.2–15.8) U/ml, respectively, $P < 0.04$. There were no differences in serum levels of AGEs or CML between patients with or without the following complications: retinopathy, cardiovascular disease, nephropathy, or abnormal autonomic function tests (RR interval).

There were no significant correlations between IVRT and RR interval deep breathing or Valsalva maneuver or the age of the patients. Neither did IVRT differ between the sexes, smokers/exsmokers versus never-smokers, or patients with or without neuropathy (data not shown). These variables were, therefore, not included in the stepwise regression analysis with IVRT as a dependent variable, although the serum levels of AGEs or CML differed within some of these variables.

Table 3—*M-mode and Doppler cardiography tests in 52 patients with type 1 diabetes*

IVRT (s)	0.09 ± 0.02
Peak E (cm/s)	0.88 ± 0.20
Peak A (cm/s)	0.68 ± 0.22
E to A ratio	1.372 ± 0.333
DT (s)	0.19 ± 0.05
Left atrium diameter (cm)	3.41 ± 0.37
Left ventricular	
Septal wall diameter during diastole (cm)	0.91 ± 0.16
Diameter during diastole (LVD) (cm)	4.74 ± 0.52
Posterior wall diameter (cm)	0.95 ± 0.17
Diameter during systole (LVS) (cm)	2.96 ± 0.50
Fractional shortening (LVFS) (%)	37.7 ± 6.7
Ejection fraction (LVEF)	0.75 ± 0.08

Data are means ± SD.

CONCLUSIONS — The finding in the present study of an association between IVRT and serum levels of AGE is in accordance with a study that showed the left ventricular diastolic compliance to be reduced in diabetic rats associated with myocardial collagen fluorescence AGEs (26). In that study, aminoguanidine, an inhibitor of AGE formation, prevented the

increase in myocardial AGE content and the reduction in diastolic compliance (26). AGEs have also been found immunohistochemically in human cardiac myofibrils (27). The lack of an association between serum levels of CML and IVRT in the multiple regression analysis is supported by the study of Khaidar et al. (3), who showed that the inhibition of heart collagen cross-linking did not modify CML levels in the *db/db* mouse. Since CML has not been found to be involved in cross-linking (7), our findings may indicate that the cross-linking properties of AGEs can have an influence on diastolic function.

Although it is well established that many patients with type 1 diabetes develop changes in left ventricular diastolic function as measured by pulse wave Doppler (1,28–30) even without coronary heart disease (9–12), the natural history of this diastolic dysfunction and its possible predictive importance for the development of ischemic heart disease and/or heart failure in diabetes is not well understood (31).

Because impaired relaxation of the cardiac muscle, expressed as an increase in IVRT, might be the first event of diastolic dysfunction, it is very interesting that we found serum levels of AGEs to be correlated with IVRT but not with the other diastolic parameters: peak E, peak A, or DT. The reason for this is unknown, but since IVRT is usually the first abnormal sign of impaired cardiac relaxation, it might be most sensitive to metabolic changes.

The presence of hypertension, heart disease, nephropathy, and autonomic neuropathy (32,33) can influence cardiac diastolic function. We found, however, a significant association between IVRT and serum levels of AGEs even when the 15 patients with cardiovascular disease were excluded from the regression analysis, indicating that the association is not a epiphenomenon occurring in patients with cardiovascular disease. Other possible confounding variables like the mean arterial blood pressure and AER were adjusted for in the multiple regression model, and autonomic nerve function tests were not found to be significantly correlated with AGEs or IVRT. Thus, although subclinical ischemia due to coronary atherosclerosis cannot be ruled out, other confounding factors have been taken into account, as discussed above.

In this study, we did not find increased serum levels of AGEs in patients with micro- or macrovascular complications, which does not rule out a possible role for AGEs in the pathogenesis of these complications. HbA_{1c}, a predictor of micro- and possibly macrovascular complications, were increased only in patients with retinopathy ($P < 0.02$), and not in patients with nephropathy or cardiovascular disease in this cross-sectional study. Interestingly, we have shown serum levels of AGEs to predict morphological changes in the kidney of patients with diabetes and microalbuminuria (13).

Smoking is a risk factor for both micro- and macrovascular complications in diabetes (34,35). Because we found increased AGE levels in present and former smokers when compared with patients who had never smoked, the long-lasting effects of tobacco on serum AGE levels was demonstrated in agreement with a recent study that showed AGEs in tobacco smoke and aqueous extracts of tobacco (23). Furthermore, serum levels of AGE–apolipoprotein B were found to be increased in nondiabetic smokers compared with nonsmokers (23). These findings might suggest that increased AGE levels increase the risk for micro- and macrovascular complications in diabetic smokers.

In conclusion, we have demonstrated that serum levels of AGEs, unlike serum levels of the non–cross-linking glycoxidation product CML, correlated positively with cardiac relaxation, indicating a role for AGEs in the increase of heart stiffness and diabetic cardiomyopathy seen in patients with type 1 diabetes, possibly mediated by the cross-linking properties of AGEs.

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