The association between glycoxidation and chronic or acute hyperglycaemia in type 2 diabetic patients with retinopathy

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Abstract
Retinopathy is the main cause of blindness in persons with diabetes and the most frequent type of diabetic microvascular complication. There are several factors considered to be retinopathy risk factors. Among them, the role of chronic hyperglycaemia and its consequence glycoxidation, has already been well established but the influence of *acute* hyperglycaemia on glycoxidation has not been fully elucidated. We have analyzed the association between daily glycaemic fluctuations (acute hyperglycaemia), estimated from plasma 1,5-Anhydroglucitol concentrations, and glycoxidation expressed as serum N^{ε}-carboxy(methyl)lysine concentrations, in type 2 diabetic patients with and without retinopathy. 1,5-anhydroglucitol concentrations in plasma were determined by an enzymatic method and N^{ε}-carboxy(methyl)lysine concentrations in serum were estimated by ELISA. After allowing for the effects of hyperlipidaemia and residual β-cell function we found no relation between 1,5-anhydroglucitol concentrations in plasma and N^{ε}-carboxy(methyl)lysine concentrations in serum and conclude that glycoxidation in patients with type 2 diabetes with retinopathy is not directly, if at all, related to acute hyperglycaemic episodes. (Int J Diabetes Metab 14: 27-31, 2006)

***Key words*:** hyperglycaemia, diabetes type 2, glycoxidation, microvascular complications, retinopathy,

Introduction

Survival among diabetic patients has been significantly prolonged by modern therapeutic regimens. Nowadays, the clinical impact of diabetes is dominated by its long-term effects on the circulatory , peripheral and other systems and organs. These late complications are the main causes of the increased morbidity and mortality in diabetic persons.

Retinopathy, the main cause of blindness in persons with diabetes, is the most frequent microvascular complication.[1,2] but many questions concerning the pathogenesis of chronic diabetic complications have yet to be fully answered.

Several factors associated with progression to retinopathy are regarded as crucial in the pathogenesis of microangiopathy, including degree of metabolic control, duration of diabetes, body mass index, blood pressure, genetic determinants, immunologic mechanisms, hormones, and growth factors.[3-6] The role of hyperglycaemia, and perhaps its associated nonenzymatic glycation and oxidation, appears to be of central importance in the development of microangiopathy.[7,8]

The consequences of chronic hyperglycaemia include increased activation of the uronic acid pathway and structural and functional changes, stimulation of the polyol pathway and changes in activity of protein kinase C, [9] stimulation of nonenzymatic glycation, and oxygen free radical production.[10,11]

Nonenzymatic glycation results in formation of covalent connections between the aldehyde group of glucose and free amino groups of proteins. The ultimate products of these reaction are advanced glycation end products (AGEs) – structurally differentiated chemical compounds. Changes in structure of collagen and intercellular matrix due to AGE formation influence the properties of connective tissue. AGEs also react with macrophage receptors, releasing cytokines and growth factors and, in this way, AGEs stimulate destructive and mitogenic processes. Moreover, the synthesis of AGE is a source of free radicals.[10,11] The toxic oxygen derivatives inhibit nitric oxide synthase and this reaction is the source of the next pool of free radicals. Reduced availability of a vasodilator such as nitric oxide could contribute to the reduced blood flow throughout small blood vessels.[10,12]

Chronic hyperglycaemia in diabetes is a major source of free radicals, which may be generated via at least three different pathways: glucose auto-oxidation, non-enzymatic glycation and activation of the sorbitol pathway.[12] Non-enzymatic glycation and oxidative reactions are closely related and termed glycoxidation.

N^{ε}-carboxy(methyl)lysine (CML) has been detected and measured in tissues and serum.[12-14] CML is a non-fluorescent, non-cross-linked structure formed as a product of both glycoxidation and lipoxidation[14] and was recommended as biochemical marker of two correlated processes: nonenzymatic glycation and glycoxidation.[12]

The role of glycoxidation in the development and progression of diabetic retinopathy has been well documented[15,16] and a correlation between glycoxidation and the incidence of retinopathy has been found in hamsters.[15-17] Microvascular complications, including retinopathy, are associated with hyperglycaemia measured as fasting glucose concentrations and HbA_1c,[18] but these parameters do not indicate acute postprandial hyperglycaemia.[19] Postprandial hyperglycaemia, defined as short-term daily glucose fluctuations, was

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recently confirmed as the most significant macroangiopathic (cardiovascular) risk factor.[20,21] The only retrospective plasma marker of such glycaemic spikes is the 1,5-anhydroglucitol (1,5-AG) concentration. The 1,5-AG concentration in plasma promptly decreases with each hyperglycaemic episode,[22,23] even short-lasting ones. 1,5-AG is eliminated by the kidneys and 1,5-AG reabsorption is competitively inhibited by glucosuria. Hyperglycaemic episodes stimulating glucosuria result in rapid 1,5-AG elimination in the urine.[22] The reference values of 1,5-AG levels in the plasma of healthy humans range between 14.4 – 30.2 mg/l, while in patients with diabetes the plasma 1,5-AG levels are markedly reduced, i.e. in patients with type 2 diabetes 1,5-AG concentrations in plasma vary between 0.9 – 26.6 mg/l, depending on the frequency and duration of hyperglycaemic episodes.[23] HbA_1c, the routine retrospective parameter of chronic hyperglycaemia, is not designed to capture acute, short-lasting, hyperglycaemic spikes.[24]

We investigated whether serum concentrations of CML are increased and linked to acute hyperglycaemia in patients with type 2 diabetes and retinopathy. To exclude the potential influence of residual β-cell function on CML concentrations [25,26] we randomized patients according to their C-peptide level.

Patients and Methods

Patients

The study group consisted of 50 patients with type 2 diabetes mellitus. A total of 28 patients (15 female, 13 male), mean age 60.9 ± 7.7 years, mean diabetes duration 9.9 ± 7.5 years with mild to moderate nonproliferative diabetic retinopathy and 22 patients (9 female, 13 male), mean age 64.4 ± 8.1 years and, diabetes duration 7.9 ± 5.9 years without retinopathy, were recruited for the study.

To enter the study, the fasting C-peptide level in each individual had to be higher than 0.5 ng/ml (ELISA Dako kit, Denmark) to confirm that basal secretory capacity of the β-cells had been preserved (reference range for healthy humans: 0.5–2.9 ng/ml). Retinopathy was diagnosed by direct ophthalmoscopy.

All patients were treated with gliclazide 40-80 mg/day (Diaprel®) or 30-60 mg/day (Diaprel MR®) and monotherapy started at least 3 months before the investigation began. All patients were treated with ACE inhibitors. Patients with a history of smoking were excluded from the study.

Among patients with retinopathy, 8 suffered from concomitant nephropathy and 20 were diagnosed with hypertension. In the group without retinopathy, arterial hypertension was diagnosed in 19 patients.

Patients had no clinical evidence of kidney or hepatic insufficiency, severe anaemia, coronary heart disease or thyroid gland dysfunction.

The study protocol was approved by the Poznan University of Medical Sciences Ethical Committee and each participant signed informed consent.

Measurements

All subjects were studied in the morning after 10 hours overnight fasting before drug administration. Measurements were made of fasting concentrations of glucose, HbA_1c, 1,5-AG, C-peptide, triglycerides, total cholesterol with HDL and LDL fractions.

Blood glucose and lipids were estimated by standard laboratory procedures.

Haemoglobin A_1c was assayed by ELISA (DAKO kit): reference range for healthy humans 4.0 – 6.0 %. 1,5-Anhydroglucitol (1,5-AG) was measured using an enzymatic method.[27,28] Fasting serum C-peptide concentrations were measured by ELISA (DAKO kit): reference range; 0.5 – 2.9 ng/ml. N^{ε}-carboxy(methyl)lysine (CML) concentrations in serum were assayed by competitive ELISA[29] with monoclonal anti-CML antibodies from ALTEON Inc.

1,5-AG measurement procedure [27,28]

The concentration of 1,5-AG in plasma was measured using a modified column enzymatic method. Briefly, 100 µl of deproteinized plasma sample was passed through a two-layer microcolumn packed with ion-exchange resin to remove glucose. 1,5-AG was efficiently recovered in the flow-through fraction. Hydrogen peroxide formed in the enzymatic oxidation of 1,5-AG with pyranose oxidase was detected by a standard method utilizing an enzymatic color-developing system. The intra-assay CV was 4.9%, inter-assay CV – 3.2% and the mean recovery was 96.6 % when estimated in our laboratory using plasma samples from 80 non-diabetic persons aged 20-60 years. Reference range included values between 14.4 – 30.2 mg/l.

CML measurement procedure

Competitive ELISA assay was conducted as described by Makita[29] modified to use anti-CML monoclonal antibody (4G9, Alteon Inc, Ramsey, NJ). Briefly, Nunc multiwell polystyrene plates were coated with BSA – AGE (bovine serum albumin – AGE) (3µg/well), populated with CML epitopes, and incubated overnight at 4 °C. Next, the coating solution was removed and the wells were washed 6 times with 200 µl of a wash buffer containing a detergent – Tween 20, then blocked with 200 µl blocking buffer in PBS for 1 h. After 6 rinses with washing buffer, 50 µl of competing antigen in dilution buffer was added, followed by 100 µl of anti-CML antibody (4G9) diluted in PBS buffer containing BSA and Tween 20. Plates were incubated at room temperature for 2 h with gentle agitation in a horizontal position. Wells were then rinsed 6 times with washing buffer. 100 µl of anti-mouse IgG antibody conjugated with alkaline phosphatase was added and incubated overnight at 4 °C. After rinsing (6 times) with washing buffer, 100 µl pNPP (para-nitro-phenyl phosphate) substrate was added to each well. Optical density (OD) at 405 nm was determined by ELISA reader after 30 to 60 min. Results were expressed as B/Bo, calculated as [experimental OD – background OD (no antibody)] / total OD (no competitor) – background OD]. The standard curve was plotted by a logit-log method: %B/Bo v. Alt927 ng/ml (standard antigen – an analog of CML, Alteon Inc, Ramsey, NJ). The range of the standard curve was 6 – 96 ng/ml.

Table 1: Parameters of glucose homeostasis and C-peptide levels of subjects with type 2 diabetes with or without coexisting retinopathy.

	Patients with retinopathy n = 28	Patients without retinopathy n = 22
Age (years)	60.9 ± 7.7	64.4 ± 8.1
Diabetes duration (years)	9.9 ± 7.5	7,9 ± 5.9
BMI (kg/m^2)	30.6 ± 3.3	31.1 ± 3.2
C-peptide (ng/ml)	2.1 ± 0.3	1.9 ± 0.4
Fasting glycemia (mmol/l/mg/dl)	12.4 ± 3.1 223.2 ± 55.8	12.5 ± 3.3 225.0 ± 59.4
1,5-AG (mg/l)	9.6 ± 5.1	9.9 ± 5.9
HbA_1c (%)	7.7 ± 1.5*	6.7 ± 1.4
Total cholesterol (mmol/l) (mg/dl)	6.1 ± 1.1 234.8 ± 43.6	6.3 ± 1.4 243.3 ± 53.2
HDL-cholesterol (mmol/l) (mg/dl)	1.1 ± 0.3 43.9 ± 11.2	1.1 ± 0.2 42.2 ± 9.6
LDL-cholesterol (mmol/l) (mg/dl)	3.3 ± 0.9 128.4 ± 34.9	3.4 ± 1.1 131.9 ± 42.9
Triglycerides (mmol/l) mg/dl	2.4 ± 0.8 211.4 ± 66.9	2.4 ± 0.9 210.5 ± 76.3

* -statistically significant for $p \leq 0.05$

Statistical analysis

All data are expressed as the mean ± SD and medians. Differences between groups were tested with Mann-Whitney rank test. Spearman analysis was performed to estimate the relationship between metabolic control parameters and CML. $P < 0.05$ was considered statistically significant.

Results

Patients were well matched for age and duration of diabetes (Table 1). There were no background differences in metabolic control parameters between patients with and without retinopathy, including fasting glycaemia and plasma anhydroglucitol concentration. The HbA_1c level was significantly higher in the group with retinopathy (Table 1). In summary, both groups were poorly controlled.

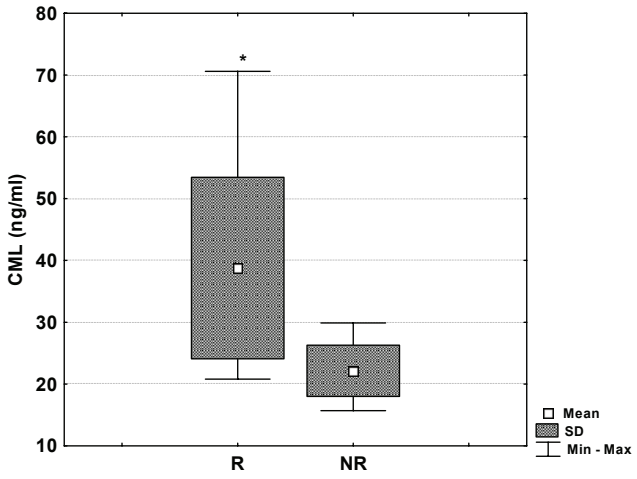

Figure 1: The concentration of N$^{\varepsilon}$-carboxy(methyl)lysine (CML) in serum of type 2 diabetic patients with and without retinopathy. * - significant difference for $p \leq 0.05$, R – with retinopathy, NR – without retinopathy

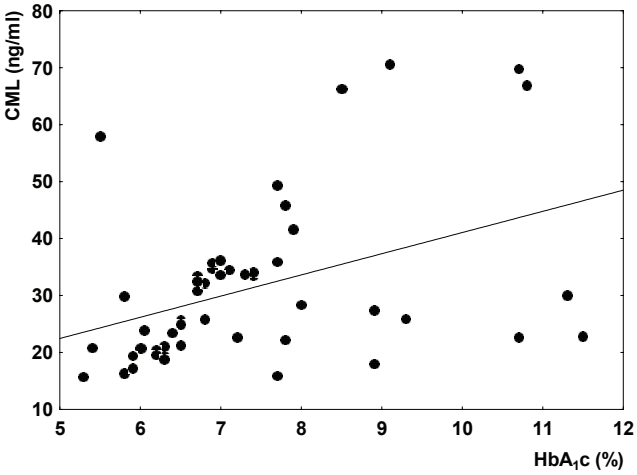

Figure 2: The relation between HbA_1c and N$^{\varepsilon}$- Carboxy (methyl)lysine (CML) in serum of type 2 diabetic patients with and without retinopathy .r = 0.4, $p \leq 0.05$

The fasting C-peptide concentrations were comparable between the two groups and mean values from both groups were included in the reference range for healthy people (Table 1).

The concentration of CML in the group with retinopathy was significantly higher than in controls (38.8 ± 14.7 ng/ml v. 21.6 ± 4.0 ng/ml) (Figure 1). The range of CML levels among patients with retinopathy was wider (20.8 – 70.6 ng/ml, median 33.9 ng/ml), than in the control group (15.7 – 29.9 ng/ml, median – 20.9 ng/ml).

A significant correlation between HbA_1c and CML (r = 0.4; $p < 0.05$) was found when both study groups were analyzed together (Fig. 2). There were no significant correlations between anhydrogluctiol, total cholesterol, HDL and LDL fractions, triglycerides and CML level .

Discussion

Results of the Diabetes Control and Complications Trial (DCCT) strongly supported the causal role of chronic hyperglycaemia in the pathogenesis of diabetic microvascular complications.[7,18] The risk of diabetic retinopathy increases stepwise with increasing degrees of hyperglycaemia. The dominant mechanism associated with chronic hyperglycaemia–dependent development of retinopathy is the formation and accumulation of advanced glycation end products (AGE) and the glycoxidation compound – CML.[12,15]

The results of the DCCT[18] demonstrated a clear link between HbA_1c as a glycaemic surrogate and the risk of retinopathy, implying a similar association between mean glycaemia and retinopathy risk. HbA_1c can be regarded as the marker of aggregate changes in blood glucose levels and correlates well only with mean plasma glucose.[19,30] The effects of acute glucose excursions on HbA_1c concentrations are limited, especially those lasting for a few hours only.[19,30] To reveal acute, short-lived hyperglycaemia, we estimated 1,5-AG levels in plasma, a sensitive indicator of short-term glucose spikes.[23]

Short-term acute hyperglycaemia (postprandial) was recently proposed to be a more significant risk factor for

macrovascular complications than chronic hyperglycaemia.[20,21] One of the mechanisms held to be responsible for tissue damage due to postprandial glycaemia is stimulation of glycoxidation.[31] There is also some evidence that acute hyperglycaemia is related to development of retinopathy.[32,33] We therefore felt it worth exploring the impact of acute hyperglycaemia on the risk of retinopathy by inducing glycoxidation and so increasing serum CML concentrations.

Although we found CML levels significantly higher in patients with retinopathy compared with those in the control group, the degree of acute hyperglycaemia (FPG, 1,5-AG) and lipid metabolism parameters (total cholesterol, HDL, LDL , triglycerides) did not differ between the groups. The FPG, 1,5-AG and HbA_1c concentrations in patients with and without retinopathy were poorly controlled, suggesting that hyperglycaemia, whether short-lived or long-term, could activate the glycoxidation process. Fasting C-peptide levels were comparable in the two groups, falling within the reference range for healthy individuals.

Our results, therefore, appear to exclude a major determinant effect of acute hyperglycaemia (FPG, 1,5-AG), lipid profile and residual beta-cell function (C-peptide) on CML concentrations in patients with retinopathy. There were statistically significant differences between groups with and without retinopathy. When both of these groups were pooled, a significant correlation was found between HbA_1c and CML levels. On the strength of these results and the well established correlation between glycated haemoglobin concentrations and diabetic microangiopathic complications,[18] it seems very likely that CML formation depends mainly on chronically raised glucose concentrations rather than acute hyperglycaemic spikes.

Other factors may be responsible for the lack of direct association between acute hyperglycaemia and CML concentration. The activity of "endogenous antioxidants" may be involved and significant differences in expression of the antioxidant genes like heme oxygenase-1, glutathione peroxidase, Cu/Zn-superoxide dismutase and Mn-superoxide dismutase have been observed in diabetic rats.[34] Moreover, it has also been suggested that CML plasma concentrations are predominantly genetically determined. [35]

In conclusion, our studies gave no support to the view that acute hyperglycaemic episodes (spikes) were directly, if at all, related to glycoxidation in patients with type 2 diabetes with retinopathy but were consistent with the hypothesis that the risk of this complication is associated with aggregate hyperglycaemia.

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