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Non-enzymatic glycosylation of urinary proteins in Type 1 (insulin-dependent) diabetes: correlation with metabolic control and the degree of proteinuria

G. Gragnoli, A. M. Signorini and I. Tanganelli

Clinical Medicine, University of Siena, Siena, Italy

Summary. In 18 control subjects and in 41 Type 1 (insulin-dependent) diabetic patients (13 with normal proteinuria, group A; 15 with microproteinuria, group B; and 13 with clinical proteinuria, group C), mean blood glucose, glycosylated haemoglobin and non-enzymatic glycosylated serum and urinary proteins, expressed as 5-hydroxymethylfurfural (5-HMF), were measured. In each group of diabetic patients, the levels of mean daily blood glucose, glycosylated haemoglobin and serum 5-HMF/mg protein were higher than in the control subjects. The urinary 5-HMF/mg proteinuria and the urinary/serum 5-HMF concentration ratio values were raised in group A and reduced in groups B and C. Moreover, they showed a negative correlation with 24-h urinary protein excretion in the control subjects and in each group of diabetic patients. The urinary 5-HMF/day in groups A, B and C was greater than in the control subjects. The urinary 5-HMF/day did not correlate with the mean daily blood glucose levels and, only in group A, did it correlate with serum 5-HMF and glycosylated haemoglobin. This suggests that, in this group, functional factors result in the increased renal elimination of 5-HMF and, therefore, of non-enzymatically glycosylated proteins. However, in the other groups of patients, this elimination depends on the degree of proteinuria.

Key words: Diabetes, proteinuria, serum and urinary glycosylated proteins, glycosylated haemoglobin, metabolic control.

It has been demonstrated recently that clinical proteinuria is largely independent of blood glucose control in diabetes, while subclinical increases of albumin and IgG urinary excretion are related to glycosylated haemoglobin (HbA_1) levels and are reversible by strict blood glucose control [1, 2].

On the other hand, experimental studies have shown that non-enzymatically glycosylated albumin (the amount of which depends on ambient glucose levels [3]) is avidly taken up by endocytosis in microvessels and may also provoke the extravasation of non-glycosylated albumin [4]. Moreover, McVerry et al. observed that renal glomerular changes, similar to those in diabetes mellitus, can be produced in mice by multiple injections of glycosylated plasma proteins [5]. Finally, other authors reported an increase in non-enzymatic glycosylation of diabetic rat glomerular basement membrane and suggested that this phenomenon might change the selective permeability of the membrane itself [6].

The aim of this study was to evaluate the non-enzymatic glycosylation of urinary proteins and its relationships with glycaemic control, proteinuria and diabetic nephropathy.

Subjects and methods

Subjects

Eighteen normal subjects, with no history of renal disease or hypertension, and 41 Type 1 diabetic patients were selected (divided into three groups according to their degree of proteinuria).

Thirteen patients had proteinuria values within 2 SD of our mean normal level (group A), 15 defined as having microproteinuria [7] had proteinuria >2 SD the mean normal value but <500 mg/24 h (group B), and 13 had clinical proteinuria (≥ 500 mg/24 h; group C). Clinical data of the subjects studied are shown in Table 1.

All the diabetic patients were insulin-dependent (ketosis-prone) at the time of diagnosis. No patients were ketoacidotic and their systolic and diastolic blood pressure values were <140 mmHg and 90 mmHg, respectively. None had evidence of non-diabetic renal disease. None was taking any therapy other than insulin. All subjects were within 10% of their ideal body weight [8]. In all cases clinical proteinuria had appeared >10 years after the diagnosis of diabetes. All these patients had evidence of diabetic retinopathy and had persistent proteinuria for at least 1 year, but not for more than 3 years before the study.

All diabetic and non-diabetic subjects had consistently negative urine cultures throughout the study.

Methods

The following variables were determined on the same day: mean daily blood glucose levels, calculated, as the mean of six values: fasting, be-

Table 1. Clinical data of the subjects studied

	Sex (M:F)	Age (years)	Duration of diabetes (years)	Plasma creatinine (μmol/l)	Proteinuria (mg/24 h)	Mean daily blood glucose (mmol/l)	Glycosylated haemoglobin (%)	Glycosylated serum proteins (nmol 5-HMF/mg)	Glycosylated urinary proteins (μmol 5-HMF/day)
Non-diabetic subjects ($n=18$)	6:12	34.7± 4.1 (21 −43)	–	79.4± 1.7 (62.6− 90.1)	62.1± 6.9 (24 − 121)	4.7± 0.1 (4.1− 5.0)	5.8± 0.2 (4.8− 6.9)	0.5 ±0.01 (0.38−0.55)	1.1± 0.1 (0.5− 2.3)
Diabetic patients									
Group A ($n=13$)	5: 8	31.9± 5.8 (18 −58)	3.7± 0.5 (1 − 7)	80.7± 2.3 (62.6− 98.3)	78.8± 7.0 (40 − 112)	8.9± 0.3[a] (7.7−11.2)	9.1± 0.1[a] (8.2− 9.8)	0.8 ±0.03[a] (0.62−1.00)	3.2± 0.2[a] (2 − 5.3)
Group B ($n=15$)	4:11	37.6± 4.8 (13 −57)	10.4± 0.9[b] (5 −15)	83.2± 2.7 (71.5−107.3)	212.7± 14.9[ab] (145 − 330)	9.4± 0.5[a] (5.7−11.7)	10.0± 0.5[a] (5.8−13.8)	1.0 ±0.07[a] (0.65−1.81)	3.8± 0.5[a] (2 − 9.2)
Group C ($n=13$)	2:11	35.1± 6.4 (21 −59)	12.3± 0.5[b] (10 −15)	82.3± 3.1 (62.6−107.3)	2499.3± 428.5[abc] (600 −6549)	9.0 ± 0.4[a] (6.4−11.2)	10.0± 0.5[a] (7.2−12.6)	1.0 ±0.07[a] (0.72−1.48)	11.8± 1.8[abc] (2.4−24.5)

Results are expressed as mean ± SEM with the range in parentheses.
[a] $p<0.05$ non-diabetic subjects versus diabetic patients; [b] $p<0.05$ group A versus groups B and C; [c] $p<0.05$ group B versus group C
Group A had proteinuria values within 2 SD of our mean normal level; group B had proteinuria > 2 SD the mean normal level, but < 500 mg/24 h; group C had clinical proteinuria ≥ 500 mg/24 h

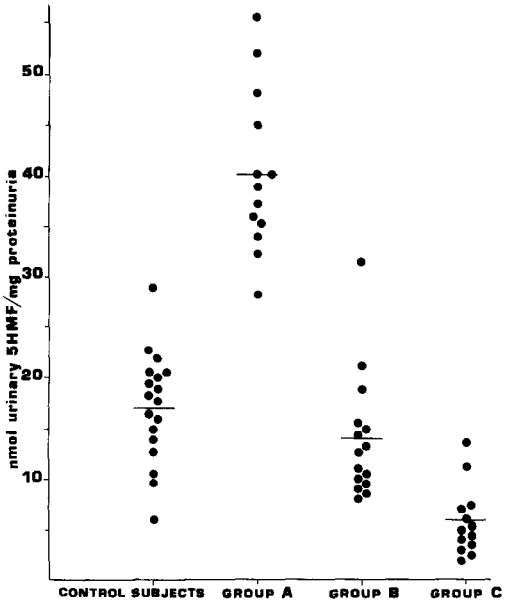

Fig. 1. Urinary 5-HMF/mg proteinuria values in control subjects ($n=18$) and diabetic patients (group A: $n=13$; group B: $n=15$; group C: $n=13$). Bar indicates the mean values for each group, with: $p<0.01$, control subjects versus groups A and C; $p<0.05$, control subjects versus group B; $p<0.02$, group A versus groups B and C; $p<0.02$, group B versus group C. (Group A had proteinuria values within 2 SD of our mean normal level; group B had proteinuria > 2 SD the mean normal level but < 500 mg/24 h; group C had clinical proteinuria, ≥ 500 mg/24 h)

fore and 2 h after meals; urinary and serum proteins; total HbA_1 levels and the non-enzymatic glycosylation of serum and urinary proteins.

Glycaemia was measured according to Trinder [9], urinary and serum proteins were assayed by the technique of Bradford [10] and HbA_1 was separated by a chromatographic microcolumn method [11]. The assay of non-enzymatic glycosylation of proteins was performed, according to Kennedy et al. [12], on fasting serum and 24-h urine collected in toluene (10 ml) at 4 °C by samples voided every 3 h to avoid fast glycosylation of urinary proteins in the bladder [13, 14]. Serum was dialyzed overnight against normal saline (1:350) and urine against distilled water (1:1000) to prevent spurious elevation of glycosylation levels due to free glucose and to fast, but labile, non-enzymatic glycosylation [12, 14]. Urine was then filtered and preserved at −20 °C until assayed. Frozen samples were thawed, filtered again and concentrated by ultrafiltration, first in cells under nitrogen with Amicon PM-10 membrane and then with Minicon (Amicon Corporation, Lexington, Massachusetts, USA) to obtain a uniform protein content in the test tubes (2.5 mg/ml) [15]. In all the samples (serum and urine), the hexoses non-enzymatically bound to the proteins by a ketoamine bond, were specifically hydrolyzed with 1 mol/l oxalic acid at 100 °C for 4.5 h and freed as 5-hydroxymethylfurfural (5-HMF). After precipitation by addition of 40% trichloroacetic acid, 1.5 ml of the supernatant was reacted with 0.5 ml of 0.05 mol/l thiobarbituric acid, incubated for 30 min at 40 °C and then for 15 min at room temperature and its absorbance at 443 nm was determined [12, 15]. All measurements were run in duplicate and an appropriate blank was subtracted from all samples. Another simultaneous reagent blank was run with samples. The calibration curve was performed with 5-HMF (Merck, Darmstadt, FRG); its concentration was determined using a molar absorption coefficient of 16.800 at 284 nm [16]. The coefficients of variation for intra- and interassay were 5.4% and 6.8%, respectively; non-enzymatic glycosylation of serum proteins was expressed as nmol 5-HMF/mg serum proteins, while the non-enzymatic glycosylation of urinary proteins was calculated as nmol urinary 5-HMF/mg proteinuria and as μmol urinary 5-HMF/day. The latter value was obtained as the product of nmol 5-HMF/mg proteinuria and 24-h proteinuria.

Statistical analyses

Results are given as mean ± SEM. Statistical analyses between groups was performed using Scheffe's test. For variables without a normal distribution, the Mann-Whitney U-test was used. Multiple regression was used to evaluate the correlation coefficients.

Results

The results showing the relationship between the 24-h urinary protein excretion and the indexes of glycaemic control [17], i.e. mean daily glycaemia, HbA_1, non-enzymatic glycosylation of serum proteins, in the non-diabetic and diabetic subjects are shown in Table 1.

Urinary 5-HMF/mg proteinuria

The urinary 5-HMF/mg proteinuria values, namely the urinary 5-HMF concentration, were elevated in

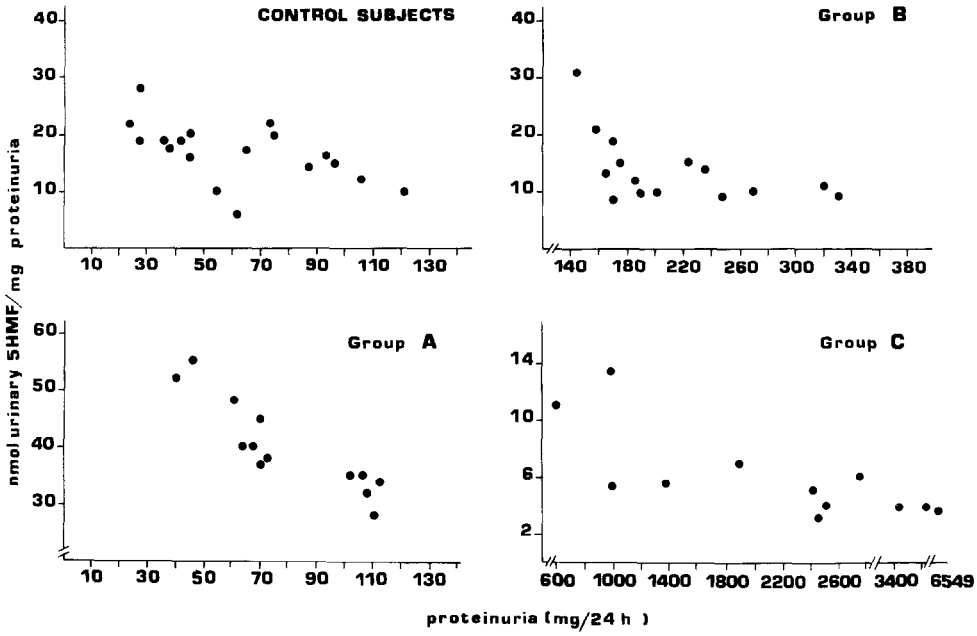

Fig. 2. Correlation of 24-h proteinuria with urinary 5-HMF/mg proteinuria in control subjects ($r = -0.544$; $p < 0.05$) and in three groups of diabetic patients: group A ($r = -0.892$; $p < 0.001$), group B ($r = -0.537$; $p < 0.05$) and group C ($r = -0.570$; $p < 0.05$). (Group A had proteinuria values within 2 SD of our normal level; group B had proteinuria > 2 SD the mean normal level, but < 500 mg/24 h; group C had clinical proteinuria ≥ 500 mg/24 h)

group A, lower than those of the control subjects in group C and diminished, to a lesser degree, in group B. Moreover, statistically significant differences were found between the groups of diabetic patients (Fig. 1).

The urinary/serum 5-HMF concentration ratio was augmented in group A (50.1 ± 4.5; $p < 0.05$) and lower than that of non-diabetic subjects (37.7 ± 2.6) in groups B and C (15.0 ± 1.7, $p < 0.001$ and 7.1 ± 1.3, $p < 0.001$, respectively). Again, significant differences were found between the diabetic groups ($p < 0.005$).

A strong negative correlation between the urinary 5-HMF concentration and the 24-h proteinuria was found in the non-diabetic subjects and in each group of diabetic patients (Fig. 2).

Equally, a significant relationship between the urinary/serum 5-HMF concentration ratio and the degree of 24-h proteinuria was demonstrated in non-diabetic subjects ($r = -0.486$; $p < 0.05$), in group A ($r = -0.839$; $p < 0.001$), in group B ($r = -0.563$; $p < 0.05$) and in group C ($r = -0.599$; $p < 0.05$).

Urinary 5-HMF/day

The urinary 5-HMF/day was significantly greater than normal in each of the diabetic groups. In group A, this clearly depended on the elevated values of the urinary 5-HMF concentration. In groups B and C, it was due to the increase both in urinary 5-HMF concentration and in 24-h proteinuria. No significant difference was found between groups A and B, whereas the values in group C were higher than those of the other diabetic patients (Table 1).

No correlation was found between urinary 5-HMF/day and the mean daily glycaemic level in the non-diabetic subjects or in groups A–C. On the contrary, highly significant positive relationships between urinary 5-HMF/day and HbA_1 ($r = 0.826$; $p < 0.001$) and with serum 5-HMF concentration ($r = 0.696$; $p < 0.01$) were found in group A. In these patients, however, HbA_1 and serum 5-HMF/mg protein showed a positive correlation with 24-h proteinuria ($r = 0.563$; $p < 0.05$; $r = 0.581$; $p < 0.05$) and a negative correlation with urinary 5-HMF/mg proteinuria ($r = -0.562$; $p < 0.05$; $r = -0.638$; $p < 0.05$).

Discussion

These data demonstrate that the three groups of diabetic patients, with similar elevated 5-HMF serum levels, showed differing 5-HMF urinary/serum concentration ratios. In group A, the ratio was increased in the presence of augmented urinary and serum 5-HMF concentrations. These could only result from a disproportionate elevation in the numerator with respect to the denominator. It suggests that in group A, apart from the serum levels, the glycosylated proteins excretion/mg proteinuria was higher than normal. In groups B and C, the differing values of the ratio were due to decreases in the numerator, because the increases in the denominator were similar. Thus, in these two diabetic groups, there was a progressive decrease in the excretion of glycosylated proteins/mg proteinuria, in spite of their overlapping serum levels.

The cause of the different, opposing urinary/serum 5-HMF concentration ratios in the normoproteinuric diabetic patients (group A) and those with micro- and macroproteinuria (groups B and C) remains to be established. However in group A, it is clear that there was, simultaneously, no significant proteinuria and an increase in the ratio of urinary/serum 5-HMF concentration. We can hypothesize that, in this group of diabetic patients, the electrostatic interaction between the charges on glycosylated proteins [18] and the glomerular membrane charges [19] may have been important.

The reduction in the 5-HMF urinary/serum concentration ratio was marked in groups B and C, and probably resulted from the degree of renal damage, judged by the degree of proteinuria in these groups. This view agrees with the finding that in all the groups examined, including the non-diabetic subjects, the urinary 5-HMF concentration and its ratio with serum 5-HMF levels demonstrated a negative correlation with the degree of 24-h proteinuria. Therefore, the degree of proteinuria is negatively correlated with the urinary glycosylated protein concentration, and when higher than normal, produces a significant decrease in the urinary glycosylated protein concentration.

Our data demonstrate, moreover, that urinary 5-HMF/day, (the product of the urinary 5-HMF concentration and the 24-h proteinuria) can discriminate diabetic from non-diabetic subjects. However, in groups B and C, the increase in 5-HMF/day is due to the increased proteinuria, while the opposite occurred in the other group of diabetic patients.

In no group did the urinary 5-HMF/day show any association with mean daily glycaemia, although it was correlated with indexes of medium and long-term metabolic control, i.e. serum 5-HMF/mg protein and HbA_1, only in normoproteinuric diabetic patients. In these subjects, the degree of 24-h proteinuria was correlated with serum 5-HMF/mg protein and HbA_1 which, in turn, showed a negative correlation with urinary 5-HMF levels. Thus, in group A, the relationship between urinary 5-HMF/day and the two indexes of metabolic control, depends on the proteinuria, which increases as glycaemic control worsens. The absence of any relationship between 5-HMF/day and the indexes of glycaemic control found in micro- and macroproteinuric diabetic patients, confirms that, in these two groups, the daily glycosylated protein excretion parallels the increase in proteinuria so that it, in turn, is not dependent on metabolic control. These data suggest that, in normoproteinuric diabetic patients only, medium and long-term metabolic control significantly affects the urinary daily excretion of glycosylated proteins and could itself be considered another indicator of glycaemic homeostasis.

The linkage between renal handling of glycosylated protein excretion found either in normoproteinuric or in micro- and macroproteinuric diabetic patients is unknown. We conclude that renal excretion of glycosylated proteins undergoes marked changes during the development of diabetic nephropathy.

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Professor Giorgio Gragnoli
Clinica Medica Università
Piazzetta della Selva 7
I-53100 Siena
Italy