Short Communication

The complexity of non-enzymatic glycation product sets of human globins

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Abstract

Aims/hypothesis. Recently an individual variability in the relationships between mean blood glucose levels and HbA_{1c} has been observed among diabetic patients. The aim of this study was to provide an accurate description and evaluation of glycated and glyco-oxidated globins from diabetic subjects and their relationship with HbA_{1c} and plasma glucose values.

Methods. We studied 20 Type 2 diabetic and 10 healthy subjects. Plasma samples were analysed by matrix-assisted laser desorption/ionisation mass spectrometry.

Results. The presence of glycated and glyco-oxidated species of both α and β globin was demonstrated. Values for these showed a good linear relationship with

HbA_{1c} values and the mean daily plasma glucose values for the 6 weeks preceding the investigation. Trends differed according to whether patients had chronic complications or not, differences being seen in the slopes of the plots relating HbA_{1c} to the abundance of glycated and glyco-oxidated species.

Conclusions/interpretation. The data obtained are consistent with the concept that individuals have a different individual proclivity for oxidation and/or that different oxidation kinetics are related to behavioural and environmental factors. Our data are thus relevant to the analysis of phenotype differences in diabetic patients.

Keywords AGE · Diabetes · Glyco-oxidated globins · HbA_{1c} · Mass spectrometry

Introduction

Several studies have demonstrated that AGE account for the more rapid tissue degeneration that is characteristic of diabetes [1]. Among glycated proteins, haemoglobin is routinely used to assess glycaemic

control of diabetic patients during the preceding 4 to 8 weeks [2]. In this context, the results of the DCCT and UKPDS studies show that diabetic complications can be reduced by good glycaemic control as monitored by fasting plasma glucose and HbA_{1c} levels. Indeed, the American Diabetes Association has published recommended levels of plasma glucose and HbA_{1c} for diabetic patients [3].

However, some studies based on the different relationships between mean blood glucose levels in the previous 30 days and HbA_{1c} values [4, 5] suggest that high- and low-glycation phenotypes exist within the diabetic population [5].

Matrix-assisted laser desorption/ionisation (MALDI) mass spectrometry is an effective tool for globin analysis [6, 7], giving evidence of the presence, to different extents, of glyco-oxidated adducts for α and β globins, which are undetectable with conventional methods of HbA_{1c} measurement. These differences

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Abbreviations: FPG, fasting plasma glucose · MALDI, matrix-assisted laser desorption/ionisation · MDPG, mean daily plasma glucose · m/3, mass to charge ratio

Table 1. Summary of results from correlation analysis of metabolic parameters and serum globins (α , β)

X variable	Y variable	Slope b ($\pm$ SEM)	Intercept a ($\pm$ SEM)	r^2	r	Significance of regression against 0	No. of data	Degrees of freedom
HbA _{1c}	FPG, all patients	20.74 $\pm$ 6.42	16.54 $\pm$ 55.15	0.3666	0.6055	$p < 0.005$	20	18
HbA _{1c}	FPG6, all patients	36.63 $\pm$ 6.41	-101.50 $\pm$ 55.02	0.6447	0.8029	$p < 0.0001$	20	18
HbA _{1c}	MDPG, all patients	41.69 $\pm$ 3.93	-140.79 $\pm$ 33.72	0.8622	0.9285	$p < 0.0001$	20	18
HbA _{1c}	β , all patients	0.0041 $\pm$ 0.0022	0.0251 $\pm$ 0.0186	0.1631	0.4038	NS	20	18
HbA _{1c}	β , patients and healthy subjects	0.0028 $\pm$ 0.0014	0.03668 $\pm$ 0.01041	0.1303	0.3610	$p = 0.05$	30	28
HbA _{1c}	$\alpha + \beta$ total, all patients	0.0151 $\pm$ 0.0024	0.0532 $\pm$ 0.0105	0.6854	0.8279	$p < 0.0001$	20	18
HbA _{1c}	$\alpha + \beta$ total, patients and healthy subjects	0.0158 $\pm$ 0.0014	0.0461 $\pm$ 0.0111	0.8080	0.8989	$p < 0.0001$	30	28
HbA _{1c}	$\alpha + \beta$ total, patients with complications	0.0229 $\pm$ 0.0050	-0.0088 $\pm$ 0.0450	0.7179	0.8473	$p < 0.005$	20	18
HbA _{1c}	$\alpha + \beta$ total, patients without complications	0.0121 $\pm$ 0.0020	0.076 $\pm$ 0.015	0.8407	0.9169	$p < 0.0001$	20	18
FPG	$\alpha + \beta$ total, all patients	0.00037 $\pm$ 0.00009	0.1085 $\pm$ 0.0177	0.4978	0.7055	$p < 0.0005$	20	18
FPG6	$\alpha + \beta$ total, all patients	0.000298 $\pm$ 0.000064	0.1199 $\pm$ 0.0141	0.5323	0.7296	$p < 0.0003$	20	18
MDPG	$\alpha + \beta$ total, all patients	0.00035 $\pm$ 0.00005	0.1057 $\pm$ 0.0104	0.7612	0.8725	$p < 0.0001$	20	18

r^2 : coefficient of determination; r : correlation coefficient (square root of r^2); FPG, fasting plasma glucose on day of study; FPG6, fasting plasma glucose in previous 6 weeks; MDPG, mean daily plasma glucose calculated over 6 weeks

may be related to the individual variability already described [4, 5], allowing a better definition of the physio-pathological state of different subjects. For these reasons, we studied metabolic and MALDI data obtained from healthy subjects and diabetic subjects with and without chronic complications.

Subjects, materials and methods

Subjects. We assessed 20 Type 2 diabetic patients (10 men, 10 women; mean age [$\pm$ SD]: 65 $\pm$ 10 years; mean disease duration: 12 $\pm$ 13 years) and 10 age- and sex-matched healthy control subjects with normal glucose tolerance. All patients were being treated with diet and 15 of them were taking hypoglycaemic drugs and/or insulin. On the day of the study, blood samples were taken for evaluation of fasting plasma glucose (FPG) and HbA_{1c} in a fasting state. The patients' plasma glucose levels (FPG and 1-hour post-prandial glycaemia) had been monitored at home for 6 weeks before the study, allowing mean daily plasma glucose (MDPG) levels to be calculated.

Informed written consent was obtained from all subjects and local ethics committee approval was granted.

Methods. For MALDI analysis, blood samples were treated as already described [6, 7]. MALDI time of flight [8] spectra were obtained using a Voyager-DE PRO instrument (Applied Biosystems, Foster City, Calif., USA) that was operating in positive ion linear mode [6, 7]. The abundance of glycosylated and glyco-oxidated species of α and β globins was calculated by integrating the relative peaks and expressing them as the percentage of unglycosylated + glycosylated + glyco-oxidated sets.

HbA_{1c} was measured by HPLC [9] (Variant II; BioRad Diagnostics, Hercules, Calif., USA). The HbA_{1c} between-run CV was 0.34 (international external quality assessment pro-

gramme); the mean bias versus the DCCT reference system was 0.64.

Statistical analysis. To compare mean values between quantitative variables, Student's t test for unpaired data was used. A p value of less than 0.05 (two-tailed) was chosen as statistically significant. Linear regression analysis was calculated by the least-squares method; the significance of regression was tested against zero using the Student's t test.

Results

In diabetic patients, the mean FPG was 10.55 $\pm$ 3 mmol/l (range 5.4–17). The mean FPG and mean MDPG of the previous 6 weeks were 11.5 $\pm$ 4 mmol/l (6.8–18.5) and 11.6 $\pm$ 4 mmol/l (7.6–19.8) respectively, while the mean HbA_{1c} was 8.4 $\pm$ 1.6% (6.6–12.2). Two patients had background retinopathy, three microalbuminuria, six clinical peripheral neuropathy, and six peripheral artery disease.

A typical MALDI spectrum of the globin fraction of a diabetic subject, without chronic complications, is shown in Figure 1b. The two most abundant peaks, at mass to charge ratio (m/z) 15126 and 15866, correspond to protonated α and β globins respectively. Many other peaks were present in all the spectra and were attributed to modified globins, together with various adducts. Protonated glycosylated globins give rise to the ions at m/z 16,028 and 15,288 (β and α respectively). These two species were also present in the spectra of globin fractions from healthy subjects (Fig. 1a) but in lower abundance. Some other peaks, mainly de-

Fig. 1. Typical MALDI spectra of globin fraction in a normal subject (a), a diabetic patient without complications (b) and a diabetic patient with complications (c)

tected in diabetic subjects, are related to glyco-oxidation processes, such as those at m/z 15,225 and 15,921 (Fig. 1b, c). In diabetic subjects with chronic complications, spectra different from those described above were obtained (Fig. 1c).

Linear regressions of HbA_{1c} against glycaemic parameters are reported in Table 1. The best linear relationship was obtained with MDPG ($r^2=0.8622$).

Low linearity was observed when the percentages of glycated and glyco-oxidated β globins (range 0.025–0.11%) vs HbA_{1c} were plotted. The best result was obtained by considering the percentage of the whole set of glycated and glyco-oxidated α and β globins (range 0.10–0.26%) (Table 1).

HbA_{1c} and MALDI data from patients with and without chronic complications are reported separately. The two linear plots (Table 1) show different trends, as indicated by the slope of the two regression lines.

Discussion

Hudson and co-workers [4] have shown that the ability to glycate haemoglobin differs significantly between patients and that the relationship between HbA_{1c} levels and previous mean blood glucose is not always linear. In a further investigation [5], in Type 1 diabetic patients, a significant linear correlation between MDPG and HbA_{1c} was found, together with a wide variability in the population-wide HbA_{1c} response. These results were explained by the occurrence of high- and low haemoglobin glycation phenotypes within the population.

Apart from peaks due to protonated α and β globins, the MALDI spectra in our study showed peaks due to glycated α and β globins, confirming our previous findings [6, 7] that both globins are glycated in vivo, in contrast to the usual thinking, as reported in the literature, on the definition of HbA_{1c} and its measurement [2]. HbA_{1c} measurements represent the integral of all glycated α and β globins, as proven by the relatively good linear plot.

Further ions occur for both globins but in healthy subjects their abundance is particularly low. They are generated by dehydration-oxidation reactions on the glucose molecule condensed on the protein, and are consequently interesting as a means of evaluating the oxidative stress undergone by the subject.

The MALDI spectra of globins of diabetic patients (Fig. 1b, c) show a clear increase in the abundance of peaks related to glycated α and β globins. Glycated/oxidated species are still present, and among them is a β globin containing a glyoxal moiety (β +57 M_r) at m/z 15,921. However, there were some clear differences between different patients. Figure 1c shows that glycated α and β globins were still present, but in this case a higher number of glyco-oxidated molecules was detectable as the species α globin + 5-hydroxymethylfuran (m/z 15,225). These results are consistent with the data reported by Hempe and co-workers [5], indicating the existence of high- and low-haemoglobin glycation phenotypes. However, our results indicate that different subjects have a different proclivity to oxidation processes occurring after globin glycation.

To investigate these aspects, we first examined the possible relationships between HbA_{1c} and glycaemic values (Table 1). The best linear plot was obtained

for MDPG values in the 6 weeks before sampling ($r^2=0.8622$), confirming that HbA_{1c} represents the result of glucose concentrations present during the life of haemoglobin [10].

When compared with metabolic parameters, the MALDI data show that the best linear relationship is found with respect to the MDPG in the 6 weeks preceding the test (Table 1). The entire set of glycated and glyco-oxidated globins yielded a plot with $r^2=0.7612$. Lastly, we studied possible relationships between the MALDI data and the clinical condition of patients. Interestingly the presence or absence of chronic complications affected the slope of the linear regression in different ways (Table 1).

We suggest that differences we observed between patients with and without complications are due to a different individual proclivity for oxidation, as indicated for glycation [5], and/or to differing oxidation kinetics related to behavioural and environmental factors. The provision of an accurate description at the molecular level of the entire set of unglycated, glycated and glyco-oxidated α and β globins would be a valuable contribution towards an accurate analysis of the different phenotypes, and important for the definition of appropriate pharmacological treatments.

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