Originals

Increase in Stable Glycosylated Haemoglobin After Induction of Poor Glycaemic Control

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Summary. Eight insulin-treated diabetic patients in good glycaemic control were studied as out-patients with frequent determinations of stable glycosylated haemoglobin (HbA_{1c}) before, during and after 1 week of induced poor glycaemic control. Stable HbA_{1c} was determined by cation exchange chromatography after elimination of the labile fraction by incubation in saline (0.15 mol/l). The increase in mean blood glucose was significant on the first day of reduced insulin therapy and greatest after 1 week (6.9 ± 3.9 mmol/l above basal values). Stable HbA_{1c} increased significantly on day 7 of the reduced insulin treatment. The increase represented, on average,

0.009% of total haemoglobin per mmol/l increase in mean blood glucose per 24 h during the period of induced hyperglycaemia. After restoring insulin therapy, a significant decrease in blood glucose was achieved on day 1 and after 2 days, the blood glucose level was similar to before the study. There was no significant decrease in stable HbA_{1c} within the first 2 weeks of improved glycaemia.

Key words: Glycosylated haemoglobin, Type 1 diabetes, stable HbA_{1c}, hyperglycaemia.

Glycosylated haemoglobin (HbA₁ or its main fraction HbA_{1c}) is considered to reflect the integrated blood glucose concentration during the preceding 1–2 months [1, 2]. The relationship between changes in glycaemic control and HbA₁ has not been well characterized in clinical practice. Recent studies have shown changes in HbA₁ level to occur within hours of changes in blood glucose concentrations [3–5, 17].

Rapidly formed HbA₁ is labile and can be removed by incubation of the erythrocytes in saline for a few hours [4, 5], by dialysis of haemolysates [18, 21] or by chemical means [19]. The influence of changes in blood glucose level hampers the value of HbA₁ as an index of average glycaemia and therefore the labile fraction should be eliminated when the assay is used for this purpose [4–6, 20]. The resulting stable fraction thus assayed is regarded chemically to be the ketoamine form [4].

There are only two clinical studies of the change in HbA₁ during induced hyperglycaemia [7, 8]. In one of these [7], the method used included the labile fraction, and in the other [8], two different methods were used and the change in HbA₁ and other indices of glycaemic control were inconsistent.

In one clinical experiment the increase in stable HbA_{1c} was determined during 24 h of hyperglycaemia

monitored by the 'artificial pancreas' (Biostator). The calculated average rate of increase was 0.006% of total haemoglobin per mmol/l glucose per 24 h [11].

Most investigators propose determination of total HbA₁ when column chromatography is used because the advantage of determining HbA_{1c} is small considering the difficulties of HbA_{1c} assay with column chromatography [9, 10]. However, when studying the kinetics of glycosylation and deglycosylation it is of interest to determine HbA_{1c}, which is the principal glycosylated fraction.

The aim of this study was to investigate the rate of increase in stable HbA_{1c} during 1 week of poor glycaemic control.

Patients and Methods

Patients

Two female and six male insulin-treated diabetic patients were studied (Table 1). All were ambulant, none was obese and none had retinopathy, neuropathy or nephropathy. The patients had good glycaemic control as indicated by basal values of mean blood glucose and stable HbA_{1c} (Table 2). The protocol for this study was approved by the local Ethical Committee and informed consent was obtained from all patients.

Table 1. Clinical details of patients studied

Patient	Sex	Age (years)	Body weight (kg)	Duration of diabetes (years)	Fasting C-peptide on day 7 (pmol/ml)	Insulin dose (units)	
						Usual	During period of poor control
1	M	28	74	10 months	0.14	26 NPH 2 soluble	0
2	M	22	63	7 months	0.16	18–20 NPH 4 soluble	0
3	F	39	58	3	0.10	24 NPH	12 NPH
4	M	23	61	2	0.28	22 Monotard 4 soluble	0
5	M	26	63	3	0.08	14–16 Monotard	8 Monotard
6	M	25	78	5	0	40 NPH 6 soluble	24 NPH 6 soluble
7	M	53	82	5	0.52	20 NPH	8 NPH→0
8	F	33	48	6	0	34 NPH	22→24 NPH

The study period consisted of 1 month during which the patients continued their normal daily activities. On testing days, they collected 24 h urine samples and measured their blood glucose themselves in the morning, before lunch, before dinner and at bed-time. The next morning after an overnight fast they came to the out-patient clinic between 07.00 and 08.00 h to deliver the blood glucose results and urine specimens and for blood sampling. During days 1–7, glycaemic control was assessed three times on three different days while the patients continued their usual treatment regime. During days 8–14, insulin was reduced as indicated in Table 1 with no other changes in treatment. The intention of insulin reduction was to obtain a blood glucose concentration of approximately 15 mmol/l without ketosis. Plasma was also sampled for C-peptide determination (day 7). The patients visited the out-patient clinic daily except on days 13 and 14 (Saturday and Sunday) and they tested their urine daily for ketones. Insulin was increased on day 15 and during the last 2 weeks, glycaemic control was assessed four times. Fasting blood glucose and HbA_{1c} were taken at each visit to the out-patient clinic. On days 7, 14, 22 and 31, fasting blood was taken for haemoglobin, reticulocyte count and haptoglobin. Fasting samples were taken at the end of the day indicated.

Methods

The BM-Test-Glycemie 1–44 (Boehringer, Mannheim, FRG) was used for self-monitoring of blood glucose [12]. Whole blood and urinary glucose concentrations were measured by an automated hexokinase method. C-peptide was determined by radioimmunoassay (Novo, Copenhagen, Denmark).

Blood samples for measuring HbA_{1c}, collected in tubes containing EDTA, were placed at 4 °C within 1 h and then washed and incubated on day 1 as described below. Haemolysates from day 10 were not prepared according to instructions and were excluded.

HbA_{1c} was determined with an ion exchange column method [14] modified from the reference method of Trivelli et al. [13] and from Gabbay et al. [2]. In this method the red cells are washed three times before haemolysis and chromatography. This step has been shown to eliminate part of the labile fraction [15]. In this study an incubation step was added to eliminate all the labile fraction. After washing in saline (0.15 mol/l), the red cells were suspended overnight in saline at 37 °C with glucose (2 mmol/l) to prevent haemolysis. The low glucose concentration was shown in a preliminary experiment not to influence the results (not presented). The specimens were haemolysed according to Gabbay et al. [2] and stored in –70 °C until assayed 1–8 weeks later. All specimens were analysed twice with an interval of

1 month between the two runs and the means of the results were used. All specimens from one individual patient were assayed in the same run and one specimen each of high and low controls from frozen haemolysates were run in parallel. Inter-assay coefficient of variation during the study was 3.0% for the high and 6.6% for the low controls. Room temperature was 21.8 ± 0.2 °C (mean ± SD) (range 21.6–22.0 °C) and buffer temperature 21.3 ± 0.4 °C (range 20.5–21.6 °C). Osmolality of the buffer was 99.3 ± 5.0 mmol/l and its pH was kept within ± 0.02. HbA_{1c} obtained from 20 non-diabetic adults (non-incubated) was 6.1 ± 1.4% (mean ± 2 SD). Statistical significance was assessed by Student's t-test for paired observations and by Wilcoxon's non-parametric test when indicated.

Results

All patients were able to adhere to the plan of the study. Table 2 shows the mean values of the variables analysed. There was a significant increase in mean blood glucose ($p < 0.001$), fasting blood glucose ($p < 0.01$) and urinary glucose (Wilcoxon $p < 0.05$) on day 1 of the reduced insulin treatment. The maximal increase in mean blood glucose was 6.9 ± 3.9 mmol/l (mean ± SD) reached on day 7 of insulin reduction (day 14). The increase in fasting blood glucose was 4.9 mmol/l, and for urinary glucose 363 mmol/24 h.

Stable HbA_{1c} did not increase significantly during the first 4 days, but was increased after 1 week of reduced insulin ($p < 0.05$). The rate of increase in stable HbA_{1c} was calculated in relation to the increase in mean blood glucose in the individual patients. The mean of these values was 0.009% of total haemoglobin per mmol/l glucose per 24 h.

After restoring insulin therapy on day 15, there was a significant decrease in mean blood glucose on the same day ($p < 0.01$) reaching the pre-study level after 2 days. At the end of the study, the mean blood glucose was lower than at the start ($p < 0.05$). Fasting blood glucose was decreased on day 3 of the increased insulin

Table 2. Blood glucose, urinary glucose, stable HbA_{1c}, haemoglobin, reticulocyte count and haptoglobin in eight insulin-treated diabetic patients

Days of study	1	3	7	1+3+7 3	8	9	10	11	12	13	14	15	16	17	22	31
Mean blood glucose (mmol/l)	6.3 ± 0.7	5.6 ± 0.5	8.5 ± 1.1	6.6 ± 0.6	10.1 ^{aaa} ± 0.5	10.7 ± 1.2	12.4 ± 1.5	10.9 ± 1.1	12.9 ± 1.6	13.5 ± 1.9	13.5 ± 1.7	9.6 ^{bb} ± 1.8	8.1 ± 1.5	7.3 ± 1.0	6.5 ± 1.0	5.2 ^c ± 1.0
Fasting blood glucose (mmol/l)	7.0 ± 1.0	7.2 ± 0.9	7.6 ± 0.8	7.3 ± 0.7	11.4 ^{aa} ± 1.4	11.1 ± 1.7	10.3 ± 0.9	12.2 ± 1.5			12.1 ± 1.6	9.9 ± 1.6		7.4 ^{bb} ± 1.0	10.4 ± 1.4	7.2 ± 1.2
Urinary glucose (mmol/24 h)	19 ± 15	9 ± 3	71 ± 30	34 ± 11	134 ^{aa} ± 42	170 ± 83	265 ± 111	225 ± 112	379 ± 153	291 ± 115	397 ± 103	134 ^{bb} ± 63	131 ± 86	60 ± 33	63 ± 47	26 ± 15
Stable HbA _{1c} (%)	6.28 ± 0.43	6.52 ± 0.50	6.33 ± 0.45	6.38 ± 0.45	6.36 ± 0.42	6.49 ± 0.46		6.61 ± 0.39			6.75 ^{cc} ± 0.37	6.70 ± 0.41		6.82 ^{aa} ± 0.50	6.84 ^{aa} ± 0.43	6.74 ± 0.46
Haemoglobin (g/l)				154 ± 2.4							148 ^c ± 2.3			146 ^c ± 2.4		146 ^{ccc} ± 1.6
Reticulocyte count (%)				0.95 ± 0.19							1.13 ± 0.18			0.86 ± 0.17		1.06 ± 0.11
Haptoglobin (g/l)				0.69 ± 0.07							0.75 ± 0.05			0.73 ± 0.08		0.66 ± 0.08
Results expressed as mean ± SEM																
^a $p < 0.05$; ^{aa} $p < 0.01$; ^{aaa} $p < 0.001$ versus the mean of days 1–3; ^b $p < 0.05$; ^{bb} $p < 0.01$; ^{bbb} $p < 0.001$ versus day 14; ^c $p < 0.05$; ^{cc} $p < 0.01$; ^{ccc} $p < 0.001$ versus day 7																

treatment ($p < 0.005$), urinary glucose on day 1 ($p < 0.01$) and did not differ from the pre-study values thereafter.

There was no significant decrease in stable HbA_{1c} during the 2 weeks of improved glycaemic control following the increase in insulin treatment.

The haemoglobin level was slightly but significantly decreased on days 14, 17 and 31 compared with day 1 of the insulin reduction ($p < 0.02$, $p < 0.05$, $p < 0.001$, respectively). There were no changes in reticulocyte counts or haptoglobin levels. Ketoneuria $> 2+$ was observed on one occasion in one patient.

Discussion

In this study the finding of a small increase in stable HbA_{1c} after 1 week in response to moderate hyperglycaemia and the average rate of increase related to the degree of hyperglycaemia support the original experimental results of Bunn et al. [16]. In that study, HbA_{1c} was determined with the reference cation exchange column chromatography measuring stable HbA_{1c} [13]. An increase was seen over 60 days and the average rate of increase was about 0.01% of total haemoglobin per mmol/l glucose per 24 h, similar to the rate of increase observed in the present clinical study (0.009%).

When the kinetics of the ketoamine were investigated experimentally in vitro [22], the same rate of increase was obtained as in the original experiments in vivo [16]. A similar rate of increase of HbA_{1c} has also been found in one clinical experiment in which hyperglycaemia was monitored with the 'Biostator' over 24 h [11]. The same rate of increase has been theoretically calculated on the predictions of the haemoglobin glycosylation as an irreversible first order reaction [23]. During the incubation of red cells in saline and high concentration glucose in vitro an increase in stable HbA_{1c} has been reported after 24 h but not after 2 h [4].

In 13 well controlled patients Boden et al. [7] found a significant increase in HbA₁ 1 week after the withdrawal of oral hypoglycaemic therapy. The increase in HbA₁ was much greater than in the above mentioned experiments and in our study. In their study, the HbA₁ method used also determined the labile fraction and this could explain the difference. In the same study the decrease in HbA₁ after reinstitution of oral therapy was slower than the preceding increase.

In another study, we found a decrease within the first days of improved glycaemic control when HbA_{1c} was determined without the preceding elimination of the labile fraction [24]. In that study, the patients were newly diagnosed diabetic patients in poor glycaemic control and the initial values of HbA_{1c} were high. In this study, however, no significant decrease was observed when stable HbA_{1c} was determined during the first 14 days of improved glycaemic control. The pre-study level of stable HbA_{1c} was low and the increase during

the short period of monitored hyperglycaemia was small. Under these conditions and the prediction that the glycosylation is irreversible, the decrease in stable HbA_{1c} within 2 weeks of good glycaemic control would be very small and hardly detectable.

The experimental circumstances in the study resemble common clinical situations of moderate temporary hyperglycaemia, which are thus able to generate small but significant and lasting increases in stable HbA₁ levels.

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