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Non-enzymatic glycation of epidermal proteins of the stratum corneum in diabetic patients

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Abstract. A selected group of diabetic patients showed a statistically significant increase in levels of glycated proteins in the stratum corneum compared with a control group. The values of glycated proteins correlated with those of glycohaemoglobin (GHb), and in diabetic patients also with serum glucose concentrations. The values of glycated proteins (and GHb) exhibited a positive correlation with age both in a control group and in diabetic patients. The average values of glycated proteins (and GHb) were slightly higher in women than in men. Determination of glycated proteins levels of the stratum corneum can serve as a stable parameter for long-term monitoring of the course of non-enzymatic glycation in structural and connective tissues and thus also for the prognosis of the development of dermatological complications related to diabetes mellitus. In vitro incubation of stratum corneum proteins and keratin with glucose resulted in an increase of their glycation. The values of glycated proteins and glycated keratin increased proportionally to the glucose concentration and duration of incubation. Glucose binding to keratin and proteins of the insoluble stratum corneum fraction appeared to occur at practically the same rate, and it is a first-order reaction with regard to the glucose concentration. Water-soluble proteins of the stratum corneum undergo non-enzymatic glycation preferentially (on average, 83.4% of the total amount of glycated proteins is present in the soluble fraction), regardless of the initial content of glycated proteins in the sample. The content of glycated soluble proteins of a higher molecular weight significantly increased after 4 weeks of incubation with glucose.

Key words: Non-enzymatic glycation – Epidermal proteins – Stratum corneum – Diabetes mellitus

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Introduction

Non-enzymatic glycation of proteins occurs in vivo after long-term exposure of proteins to higher glucose concentrations. The essence of this modification process is a chemical reaction between a carbonyl group of glucose and a free amino group of the protein, generally called the Maillard reaction [1].

In connection with the pathogenesis of diabetes, the above-mentioned modification reaction was first reported when glycohaemoglobin (GHb) was discovered [2]. Increased non-enzymatically glycated Hb concentrations in diabetes mellitus [3, 4,] led to intensive research into similar excess glycation of other tissue proteins, especially in an attempt to establish a link between this process and the chronic complications of diabetes mellitus. Since then, the non-enzymatic glycation of a number of proteins from all tissues of the human organism has been demonstrated in healthy subjects and particularly in diabetic patients [5].

At present, non-enzymatic glycation seems to be only the first step of a complex sequence of Maillard reactions, referred to in ageing individuals as "non-enzymatic browning" [6]. In time, the Amadori products of glycation gradually undergo a series of sequential reactions and changes resulting in the formation of "advanced glycation endproducts" (AGE) [7]. These changes include protein denaturation and polymeration, formation of rigid cross-linked structures and generation of specific protein-bound fluorophores. These adducts are associated most often with long-lived proteins such as lens crystallin, collagen and myelin and have also been detected in vivo [6–11].

It has been shown that keratin proteins also undergo non-enzymatic glycation [12–14]. The aim of the present work is to follow the glycation of keratin and other proteins of the stratum corneum, and thus contribute to the understanding of the course of modification reactions in tissues, their consequences and potential relation to the development of skin disorders in dermatological patients with diabetes.

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Materials and methods

The study group included 148 diabetic patients being treated at the 1st Department of Dermatovenerology in Brno for various diseases, mainly leg ulcerations and microbial eczema. The group comprised 78 women and 70 men aged 19–85 years. Patients were divided into four groups according to the character of their diabetes: (1) type I (DM I), (2) type II (DM II), (3) first diagnosed during hospitalization (DM n) and (4) impaired glucose tolerance (IGT). A group of 50 patients (25 men and 25 women) who evidently did not suffer from a saccharide metabolism disorder was used as the control group.

The stratum corneum specimens were taken from the sole surface area, without applying any external therapy, using a dermatological shaver. On average, a specimen of 300 mg stratum corneum was taken from each patient. The specimen was dried to constant weight (2 h, 60 °C) and homogenized (Retsch). Weight losses related to drying were within 7%–13% of the initial specimen weight (9.5% on average).

Simultaneous with the stratum corneum specimen, a blood sample was taken for the determination of GHb.

Assays

The serum glucose concentration was determined by the enzyme photometric method using the Oxochrom glucose diagnostic kit (Lachema). Hb was determined colorimetrically using the glycosylated haemoglobin diagnostic kit (Lachema). To analyse protein concentration in the stratum corneum and in the fractions obtained by chromatography, the colorimetric method according to Lowry et al. [15] or the measurement of absorbance at 280 nm was used.

Measurement of non-enzymatic glycation

Sample preparation. For the usual determination of total glycated proteins of the stratum corneum (GProt), a sample of 50 mg dried homogenized preparation of the stratum corneum was suspended in 1.5 ml solvent (distilled water or buffer if the sample was chromatographed: gel filtration, 0.01 mol/l phosphate buffer, pH 7.4; affinity chromatography, 0.05 mol/l TRIS-HCl buffer, pH 8.1). Before separation of the soluble fraction, the stratum corneum specimen was suspended in a solvent (see above) for a period of 1 h while shaken intermittently and then centrifuged. The soluble fraction (supernatant) was used directly for analysis while the insoluble fraction was washed several times in a solvent. When determining the total GProt or pure keratin, the assay was performed directly after suspending.

Colorimetric determination of glycated proteins. Our own modification of a colorimetric method [16] based on the detection of a yellow product generated by a reaction of 5-hydroxymethylfurfural (5-HMF) and thiobarbituric acid [17] was used for the determination.

Treated samples were hydrolysed with 85% phosphoric acid (1 h, 100 °C). After cooling, ballast proteins were separated from the sample by precipitation with cool trichloracetic acid (2.45 mol/l), followed by centrifugation. The supernatant was then used for the colour reaction with thiobarbituric acid (2.5 mol/l) in a ration of 2:1. After 40 min at 30 °C in a water bath, the colour intensity was determined photometrically at 443 nm (Cary 118 spectrophotometer; Varian). Each series analyzed contained a control sample of the standard GProt value, fructose calibration solutions (0.1–0.3 mmol/l) and a blank. The amount of GProt was expressed in μmol of fructose per gram of dry weight.

Affinity chromatography. A column filled approximately with 10 ml boronic acid agarose gel was equilibrated with 20–30 volume units of a starting buffer (0.05 mol/l TRIS-HCl, pH 8.1, with 0.02% NaN_3). Elution of GProt was accomplished using 0.3 mol/l sorbitol in 0.05 mol/l TRIS-HCl buffer, pH 8.6, with 0.02% NaN_3. The flow rate was adjusted at 25 ml/h. In the 2.5-ml fractions the protein concentration was determined, and the presence of GProt was detected colorimetrically. Affinity chromatography was used only for the determination of soluble GProt.

Characterisation of proteins

Gel filtration. A sample of soluble GProt (usually 175–200 mg dry weight in 3.5 ml buffer, i.e. approximately 40–60 mg protein in 0.01 mol/l phosphate buffer, pH 7.4) was applied to the column K 16/40 (Pharmacia) filled with about 70 ml Sephadex G-15 and equilibrated with 20–30 volume units of the same buffer. Separation continued at a constant flow rate of 0.4 ml/min. The eluate was collected into 2-ml fractions.

Free amino groups determination. Separated soluble GProt from the stratum corneum extract and also joint fractions from gel filtration were examined for free amino groups. Samples were treated with 2.4-dinitrofluorobenzene, and groups identified by thin-layer chromatography [18].

Electrophoresis PAGE-SDS. The joint lyophilized fractions from gel filtration were used as samples. The optimized SDS-polyacrylamide gel electrophoresis [19, 20] using a gradient of *N,N′*-methylene-bisacrylamide (0.5%–3%) was carried out in a protein double slab electrophoresis cell (Bio-Rad). Electrophoresis conditions were: gel gradient of 8%–21%, gel surface of 0.15 × 14 cm, separation time of 6.5 h at constant current of 25 mA. LMW calibration kit (Pharmacia) was used as the molecular weight standard. The protein zones were stained with Coomassie blue R-250. The electropherograms were analysed in a Vitatron densitometer.

In vitro glycation

A simple model systems for studying the non-enzymatic glycation of pure keratin and a mixed preparation of the stratum corneum homogenate of an initial value of total GProt = 3.2 μmol F/g dry weight was used.

Keratin or stratum corneum (50 mg) was incubated in a suspension of 0.01 mol/l phosphate buffer, pH 7.4, containing antibiotics and NaN_3 with glucose in a concentration range of 0–30 mmol/l at 10 °C. In samples the initial value of GProt and the values after 1, 2, 3, 4 and 8 weeks of incubation were determined. Free glucose was removed from the incubation mixture for keratin and insoluble GProt by washing it several times with distilled water before the determination. The determination of GProt or glycated keratin was performed colorimetrically. From separated soluble GProt, free glucose was removed by ultrafiltration using the membrane filter YM 05 (Amicon). The soluble GProt level was determined using both a colorimetric method and affinity chromatography as an orientation method. A chromatographic analysis of soluble GProt at 1-week intervals of incubation was done by gel filtration.

Statistical methods

The values of GHb, glycated proteins and glucose in diabetic patients and a control group were compared using the *t*-test. Correlations between the values of individual parameters were calculated using the Spearman correlation coefficient.

Chemicals

Purified keratin from cornea, insoluble in water, was purchased from Merck. Boronic acid agarose was the product of ICN Biomedicals (UK). Sephadex G-15 as well as LMW calibration kit L 14

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(4000–94 000) were from Pharmacia (Sweden). Other materials for electrophoresis separation were mostly from Serva, Heidelberg (Germany). Membrane filter YM 05 was obtained from Amicon (USA).

Results

Evaluation of a group of patients

Table 1 presents the mean values of GHb, glycated stratum corneum proteins (total) and serum glucose concentrations in a control group and in individual groups of diabetics.

The highest mean values of GProt were observed in patients with recently discovered and so far non-compensated diabetes. A normal range of GProt values was determined on the basis of evaluating the results of a control group as 1.5–3.2 μmol F/g dw.

Within the group of patients evaluated, a positive correlation was found between the values of GHb and total GProt (at a significance level of 1%), particularly in diabetic patients ($r=0.682$) and in a control group ($r=0.579$). In a group of diabetic patients, correlation was also found between the values of GProt and serum glucose ($r=0.545$).

Table 1. Glycohaemoglobin, glycated proteins of the stratum corneum and serum glucose measurements

Group	*n*	Glyco-haemoglobin		Glycated proteins		Glucose	
		x̄	SD	x̄	SD	x̄	SD
Control	50	4.9	0.6	2.5	0.5	4.7	0.5
DM I	12	9.3	1.4	3.9	0.8	12.6	6.6
DM II	98	7.8	1.3	3.6	1.3	10.1	2.8
DM n	25	8.4	2.7	4.3	1.5	12.0	4.9
IGT	13	5.2	0.4	2.8	0.7	6.6	1.1

DM I, Type I diabetes mellitus; DM II, type II diabetes mellitus; DM n, diabetes mellitus first diagnosed during hospitalization; IGT, impaired glucose tolerance [pathological OGT (T)]

Table 2. Correlation between protein glycation and patient's age and sex

Group	*n*	GHb			GProt		
		x̄	SD	*r*	x̄	SD	*r*
Women	103						
Control	25	5.18	0.73	0.709	2.78	0.87	0.690
Diabetic	78	8.37	1.16	0.710	3.76	1.09	0.569
Men	95						
Control	25	5.11	1.21	0.708	2.65	0.56	0.930
Diabetic	70	7.25	0.98	0.630	3.68	1.21	0.763
Whole group	198						
Control	50	5.15	0.68	0.889	2.62	0.67	0.872
Diabetic	148	7.81	1.54	0.662	3.77	1.14	0.628

r, Correlation coefficient (with age)

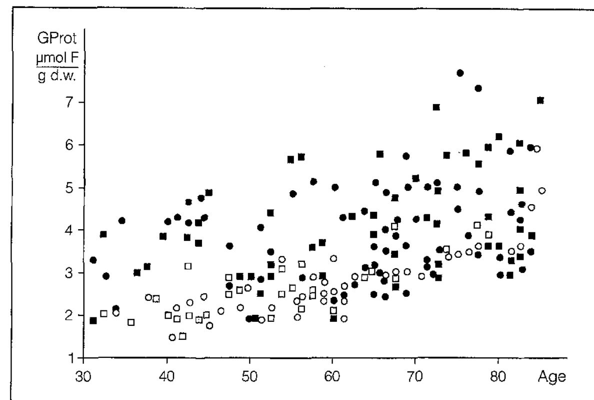

Fig. 1. Glycated proteins (GProt) versus age: ● female diabetics; ■ male diabetics; ○ female control group; □ male control group

The dependence of GHb and GProt levels on age was followed both in the whole group of diabetic patients and in a control group, and in groups of men and women control and diabetic patients. The results obtained including the correlation coefficients (evaluated at a significance level of 1%) are summarized in Table 2. Figure 1 illustrates graphically the dependence of GProt values on the patients age.

Slight differences in GHb and GProt levels were observed between women and men in the control group, while the differences between these values were somewhat higher in diabetics.

In both groups (male and female) as well as in the whole group of diabetic patients and also in a control group, a good correlation was observed between GHb and GProt values and the subjects' age (see Table 2).

Characterisation of glycated proteins

Distribution between soluble and insoluble fractions. The amount of total GProt was determined in 12 selected specimens of the stratum corneum. The soluble fraction contains on average 83.4% of the total amount of GProt regardless of whether the specimen came from a healthy individual or a diabetic patient with a higher level of protein glycation.

Gel filtration. High-porous (Sephadex G-100) and low-porous (Sephadex G-50, G-25, G-15 and G-10) gels were tested in preliminary experiments. Sephadex G-15, enabling the most efficient course of separation of predominantly low-molecular weight (LMW) proteins of the tissue examined, was selected for the chromatographic analysis. Figure 2 compares the elution profiles of the samples with normal and pathological initial values of GProt in a series of samples with increasing GProt values. From the chromatograms it is evident that glycated proteins were separated into three larger and two smaller groups according to their molecular weight. Maximum IV and V are detectable only in samples with higher total GProt

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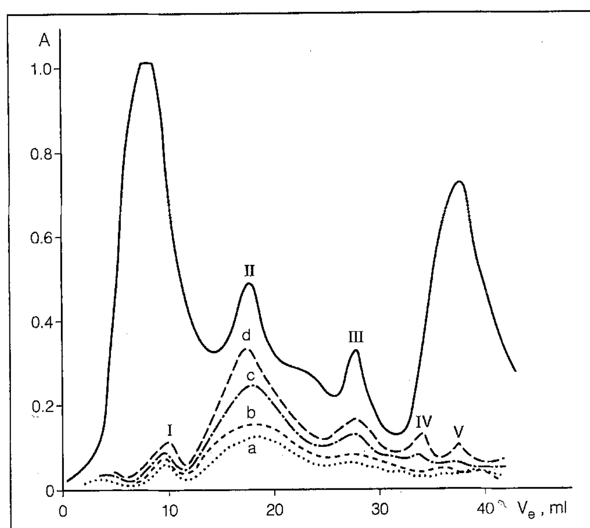

Fig. 2. Chromatography of samples with an increasing content of total GProt using Sephadex G-15. Samples: 30–50 mg of proteins, raw extract applied. V_e, Elution volume; *solid line*, profile of protein concentration in the eluate; *a, b, c, d*, glycated proteins in fractions; $GProt_{total}$ = 2.2 (*a*), 2.8 (*b*), 4.5 (*c*) and 6.0 (*d*) µmol F/g dw; *I–V*, groups of glycated proteins with gradually decreasing molecular weight. *Note:* Profiles of protein concentration for individual samples are similar. Therefore, only one shown for illustration (each sample is from another patient)

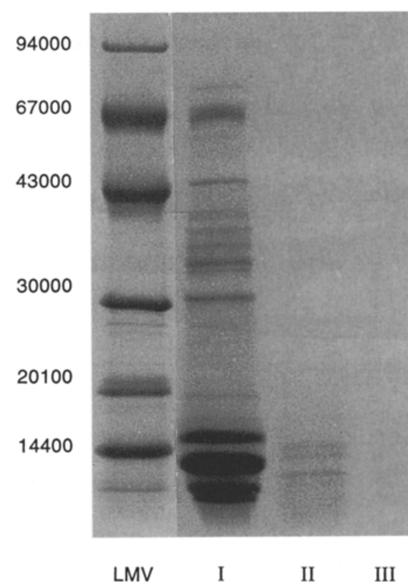

Fig. 3. PAGE-SDS electrophoresis: *I, II, III*, Groups of proteins in the same order in which they are eluated from Sephadex G-15; *LMW*, calibration set of standards of low molecular weight

Table 3. Determination of free amino groups in soluble fraction of the stratum corneum extract

Sample	Before hydrolysis (µmol $-NH_2$/mg dw)	After hydrolysis (µmol $-NH_2$/mg dw)
Crude extract	5.50	2.03
Soluble GProt		
Fraction I	1.41	0.57
II	0.94	0.65
III	0.94	0.33
IV + V	4.24	0.27

values. Proteins of the IInd maximum are glycated preferably. The increase of the peak area is linear with the increasing initial value of total GProt.

Free amino groups determination. Results are summarized in Table 3. The number of fractions corresponds to the number of fractions from gel filtration (see Fig. 2). The amount of free amino groups in samples analysed both before hydrolysis and afterwards corresponds to the previously described composition of stratum corneum extracts [21].

Molucular weight determination. PAGE-SDS electrophoresis was used for more exact determination of the molecular weight in individual protein groups obtained by gel filtration. Figure 3 shows the results, including separation of the set of LMW standards. We managed to detect only proteins related to the first two peaks (see Fig. 2), where the Ist maximum is related to a larger number of various proteins of molecular weight ranging from 10 000 to 70 000 Da. Only three clear bands corresponding to proteins of presumed molecular weight of 13 000–15 000 Da are associate with maximum II.

In vitro glycation

Figure 4 presents changes of the values of glycated proteins or keratin and their dependence on the duration of incubation in a medium with increasing glucose concentrations (within physiological range). From Fig. 4 it is evident that the values of GProt and glycated keratin increase proportionally with glucose concentration and the duration of incubation. The rate of glycation was practically indentical in both cases. Glucose binding to keratin and water-insoluble proteins of the stratum corneum is a first-order reaction with regard to glucose concentration. The value of insoluble GProt represents, on average, 16.7% of the total value of GProt regardless of the length of incubation.

In addition to a colorimetric method, the control determination of the soluble GProt fraction was done by affinity chromatography using boronic acid agarose. The GProt level was also determined in fractions colorimetrically, while evaluation of the total GProt (%) by affinity chromatography was done only from the values of A_{280} in fractions. From the first experiments it is evident that the values of soluble GProt measured colorimetrically increase somewhat faster than those obtained by affinity chromatography.

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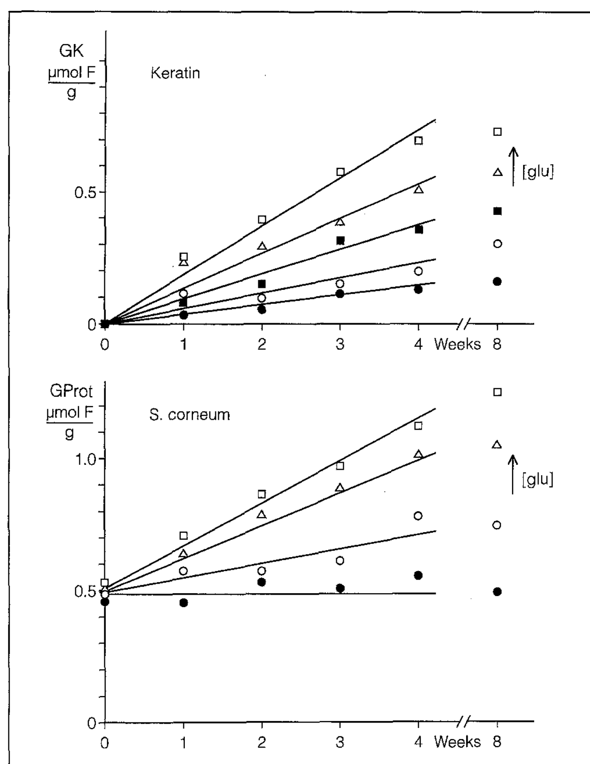

Fig. 4. Dependence of the value of glycated proteins and glycated keratin on the duration of incubation in various glucose concentrations in an incubation medium in vitro. Glucose concentration in medium: 0, 5 (●), 10 (○), 15 (×), 20 (△) and 30 (□) mmol/l

From the results of chromatographic analysis of soluble GProt after in vitro incubation with glucose, it is clear that after 4 weeks of incubation the content of GProt of higher molecular weight increased significantly (chromatograms not shown), with a corresponding decrease of LMW GProt. In all the groups followed, the portion of glycated proteins increased proportionally with the duration of incubation.

Discussion

As a result of post-synthetic modifications of the non-enzymatic glycation type, significant changes of physical and functional properties of proteins occur. Glycation of connective tissue proteins results, in addition to reduced solubility and higher thermal stability, in a higher tendency to form intramolecular cross-linkages between protein filaments, as was described for collagen [11, 22]. In the case of lens crystallin, large aggregates of extremely high molecular weight are formed [23]. A possible correlation between higher glycation, cross-linking and the incidence of neuropathic ulceration was described for keratin [12]. In fact, for various skin disorders, a higher rigidity of tissue structures was observed in diabetics patients [24, 25].

The major protein component of the stratum corneum is keratin, a fibrous scleroprotein characterized by high insolubility and resistance to enzymic lysis and autolysis. In addition, the stratum corneum contains 26.3% of water-soluble substances of which protein make up 2.4% [21]. Based on our results it is possible to conclude that the conditions for glycation in the stratum corneum are better met by soluble proteins, when compared with insoluble ones. On the other hand, irreversible modification of a keratin molecule results in more serious physiological consequences due to its long lifetime and structural and mechanical functions. According to data in the literature, glycated proteins are more reactive than non-glycated proteins (e.g. IgG binding to glycated collagen is 4 times faster than binding to non-glycated protein; [26]). Consequently, we assume that a high proportion of soluble GProt could function in the process of glycation in the stratum corneum as an available reactive potential, becoming involved in binding to keratin in late phases. This problem deserves further investigation.

From the literature dealing with glycation of hair and nail keratin [13, 14] it is evident that the level of glycated keratin can serve as a stable parameter for long-term monitoring of glycaemia and its compensation. The comparison of data on hair glycation [13] and our results obtained in a double group of patients suggests that glycation of the stratum corneum keratin can also be used for this purpose. However, we assume that use of this parameter would be more valuable to estimate the degree of glycation in tissues, and thus to predict complications related to diabetes. In dermatology, this parameter can be used to estimate the course of a disease and its healing capacity and can also serve as auxiliary data when selecting therapy.

While evaluating our patients, we found slightly higher values of GHb in women than in men, both in a control group and in diabetic patients (see Table 2). Greater differences were observed in GProt values, which were also higher in women. For the diabetic patients, neither the duration of the disease nor the kind of therapy were taken into consideration during the evaluation; however, most of them were taking insulin. We assume that the differences have practically no significance with regard to the disease. Similar data can also be found in the literature, even though the sex difference has not been explained [27].

Within our group of patients, there was a relatively good correlation between the levels of glycated proteins and GHb and age (Table 2). While the GHb concentration should not causally depend on age, a similar correlation was described in healthy people and in diabetic patients [28, 29]. In the case of GProt, a dependence on age is more likely and can be explained analogously to the reactions occurring during glycation and tissue ageing [11, 30].

Methodological approaches to the monitoring of glycation of the stratum corneum proteins are limited by the fact that the sample contains water-soluble and -insoluble fractions. To assess any sample containing an insoluble fraction, it is only possible to use a colorimetric method. This is universally applied, furthermore, its results are not influenced by a labile form. Soluble GProt can also be assessed by other methods, of which affinity chromatography is the best for the sample composition

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