Vol. 99, No. 3, 1981 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
April 15, 1981 Pages 1011-1019

QUANTITATION OF LYSINE-BOUND GLUCOSE OF NORMAL AND DIABETIC ERYTHROCYTE MEMBRANES BY HPLC ANALYSIS OF FUROSINE [ε-N(L-FUROYLMETHYL)-L-LYSINE]

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Received February 27,1981

SUMMARY: Non-enzymatic glycosylation of erythrocyte membranes was studied using a non-radioactive and sensitive procedure for specific quantitation of lysine-bound glucose in proteins. About 2 nmol lysine-bound glucose/mg protein were found in ghosts from normal erythrocytes, and this value was about doubled in diabetic patients. In vitro incubation of normal ghosts with glucose gave rise to levels of lysine-bound glucose similar to those found in diabetics. There was a linear correlation between the amount of lysine-bound glucose of total hemoglobin and of membrane proteins. Membrane glycosylation also depended on the age of erythrocytes displaying significantly higher values in old cell populations.

INTRODUCTION

The occurence of non-enzymatic glycosylation of hemoglobin has been known for a long time (1, 2), yet it was only recently that one has begun to study this type of interaction of glucose with other proteins and its possible pathophysiological consequences. Several proteins susceptible to non-enzymatic glycosylation can now be added to hemoglobin, such as serum albumin (3, 4), the lens crystallins (5), collagen (6, 7, 8), insulin (9), and renal glomerular proteins (10). Evidence for non-enzymatic glycosylation of erythrocyte membrane proteins has been presented by the demonstration of $NaBH_4$ reducible linkages (11, 12) which appeared to

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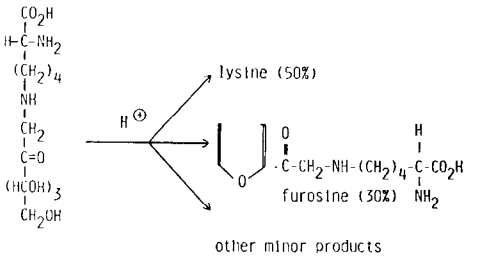

Fig. 1 Degradation products of fructose-lysine upon hydrolysis in 6 N HCl according to (14). Yields are given in brackets.

be elevated in diabetic patients (12). In the present work we have studied membrane protein glycosylation of human erythrocytes taking advantage of a new procedure developed in this laboratory for the determination of lysine-bound glucose in human serum albumin (13). It is based on the formation of [ε-N(L-furoyl-methyl)-L-lysine] (furosine), a product of acid hydrolysis of ε-aminolysyl-glucosylated proteins (14) (Fig. 1) which is measured by high performance liquid chromatography (HPLC). This assay has the great advantage of avoiding the use of radioactive (tritium) labelled material, and moreover, of yielding quantitative information on the number of glucose molecules specifically bound in ketoamine linkage to lysine residues of polypeptides.

MATERIALS AND METHODS

Preparation of erythrocyte ghost suspensions and furosine determination

Heparinized venous blood was obtained from healthy volunteers or diabetic patients by venipuncture, and erythrocyte membranes were prepared within the next four hours. Membranes were prepared starting from 2.5 ml blood essentially according to the method of Dodge (15) except that the ghosts were washed more extensively (five times). After lysis of the erythrocytes and centrifugation, the supernatant was used for estimation of ε-aminolysine-bound glucose in hemoglobin. Hydrolysis in 6 N HCl was performed as described (13).

Prior to hydrolysis the membranes were solubilized by adding 0.1 ml of 2 % sodium dodecylsulfate (SDS) solution to 1 ml of ghost suspension. In each case control samples (0.5 ml) were incubated after addition of ca. 10 mg $NaBH_4$ at 25 °C for one hour for reduction of ketoamine linkages, and dialyzed against 0.9 % NaCl solution prior to hydrolysis. The amount of ε-aminolysine-bound glucose was determined by HPLC and calculated from the peak height of furosine in the chromatograms using synthetic fructose-lysine (13, 16) as standard. Protein was determined according to the Bradford (17) or Lowry (18) method using Labtrol (Merz und Dade, München, FRG) as standard. Hemoglobin contamination of erythrocyte membranes was judged from SDS-polyacrylamide gel electrophoresis on 7.5 % gels according to Laemmli (19). Proteins were stained with Coomassie Blue, and the gels scanned with a Gilford spectrophotometer 250. Only membranes containing less than 3 % hemoglobin were analysed.

Separation of young and old erythrocytes

Heparinized blood samples from normal persons were centrifuged for 10 minutes at 600 g. The plasma and buffy coat were carefully removed by aspiration. Young erythrocytes were grossly separated from old cells by centrifugation for 60 minutes at 30 000 g at 30 °C (20). Volumes of 10 % each from the top or the bottom fractions of the packed cells (11 ml) were carefully collected. After washing the erythrocytes three times in isotonic phosphate buffer, ghosts were prepared as described above. Glutamate-oxaloacetate transaminase (GOT) (EC 2.6.1.1) was determined spectrophotometrically on an ACA analyzer (Du Pont Company, Wilmington, U.S.A.).

In vitro glycosylation of ghosts

Ghost suspensions (1 ml) prepared from 2.5 ml blood of normal persons were incubated at 37 °C in 5 ml 10 mmol/l potassium phosphate buffer, p_H = 7.4, containing increasing concentrations of glucose and 0.02 % sodium azide. After 70 hours the suspensions were washed two times with isotonic NaCl solution and analysed for furosine content as described.

High performance liquid chromatography

HPLC was carried out on a Model 600 A Solvent Delivery System, Model Wisp automated Injector and Model Absorbance Detector (all from Waters Associates, Inc. Milford, MA 01757). Two columns arranged in series were used, one 5 µ C_{18} column (20 cm x 4 mm) from Machery and Nagel (Düren, GFR), and one µ Bondapak C_{18} (30 cm x 3.9 mm) (Waters Associates, Inc.). Absorption was recorded simultaneously at 280 and 254 nm detector sensitivity set at AUFS = 0.01. As isocratic eluent 0.064 % H_3PO_4 was used. The appearance of furosine on HPLC of an acidic hydrolyzate of erythrocyte ghosts, and the disappearance of the furosine peak in the $NaBH_4$-reduced control sample is illustrated in Fig. 2 A,B.

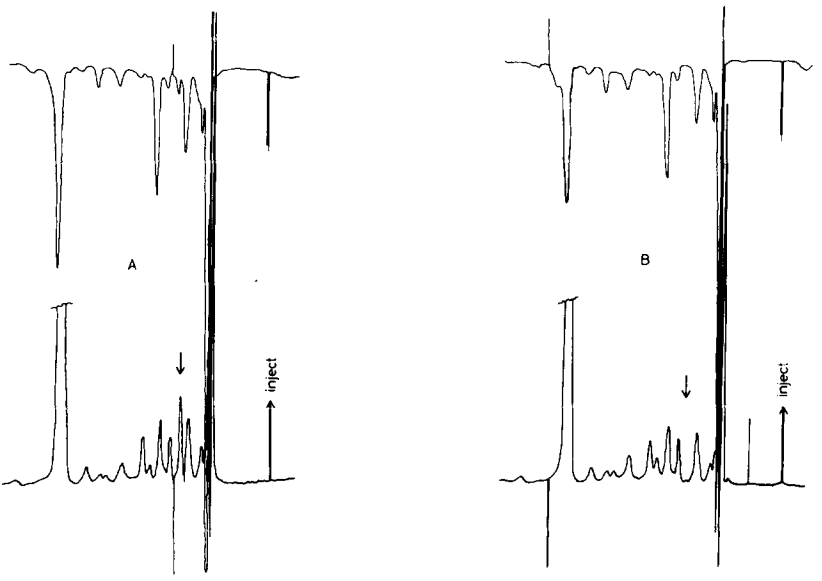

Fig. 2 Elution profile of an erythrocyte membrane hydrolysate of a diabetic patient on HPLC. The UV detector was set at 280 nm (lower) and 254 nm (upper tracing). The arrow indicates the furosine peak (A) which is missing in the control after $NaBH_4$ reduction (B). For further details see Materials and Methods.

RESULTS AND DISCUSSION

In earlier experiments we found that determination of furosine is a specific, sensitive and accurate method for quantitative investigations of glucose bound covalently to the ε-amino groups of lysine in proteins (13). In the present work this method was applied for the quantitation of non-enzymatically linked glucose to erythrocyte membrane proteins and hemoglobin of normal and diabetic persons.

Data on non-enzymatic glycosylation of erythrocyte membranes are presented in Table 1. It is shown that the membranes obtained from normal persons contain on the average about 2 nmol lysine-bound glucose per mg of protein, and that this value is about

Table 1 ε-Aminolysine-bound glucose of normal and diabetic erythrocyte membrane proteins.

	Furosine, nmol/mg protein[a)]	
	$\bar{X} \pm$ S.D.	range
Controls (n = 10)	2,16 ± 0,42	1,4 - 2,6
Diabetics (n = 9)	4,19 ± 1,99	2,7 - 8,7

a) determined according to Bradford (15). Related to Lowry protein (16) the values become 1.6 times higher. For details of membrane preparation and furosine determination see Materials and Methods section.

doubled regarding the membranes from diabetic patients. Although absolute data for comparison with our values are lacking, a similar increase in glycosylation was derived by others from the incorporation of [^{3}H] borohydride into erythrocyte membrane proteins from diabetic patients (12).

Human hemoglobin has recently been reported to have glucose bound not solely at the N-terminal valines of the β-chains like in hemoglobin A_{1c} but also at ε-amino groups of lysine residues of the α-and β-chains (21). We have observed that the hemoglobin fractions HbA_{1a+b}, HbA_{1c} and HbA_{II} contain equal proportions of lysine-bound glucose per mg of protein (determined as furosine) whether from normal or from diabetic induviduals (data not shown). In Fig. 3 we have compared the glycosylation of total hemoglobin and the membrane proteins within the same batches of normal and diabetic erythrocytes. It is clear that the increase in lysine-bound glucose of the membranes is accompanied by a corresponding increase of that of hemoglobin. A similar correlation regarding the glycosylation of HbA and HbA_1 was observed by Gabbay et al. using the colorimetric thiobarbituric acid assay

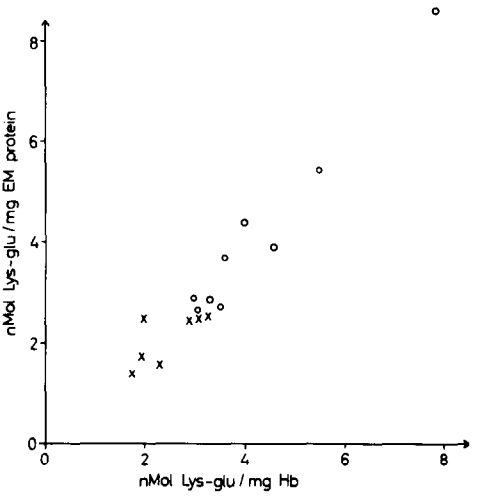

Fig. 3 ε-Aminolysine-bound glucose of erythrocyte membranes and of total hemoglobin.
x - x normal; O - O diabetic
For experimental details see Materials and Methods.

for protein-bound glucose (22). This indicates that glucose incorporation into membrane proteins and hemoglobin occurs at similar rates and likewise remains stable over the same period of time, i.e. the life span of the erythrocytes.

Like hemoglobin, membrane proteins also undergo glycosylation in vitro upon incubation of erythrocyte ghosts with glucose. There was a linear increase of lysine-bound glucose with increasing glucose concentrations. At a load of 500 mg/dl the membranes contained about twice as much covalently bound glucose molecules as at 125 mg/dl thereby falling well within the range of in vivo glycosylated red cell membranes of diabetic patients (see Table 1).

Decreased deformability of erythrocytes has been reported in diabetes mellitus (23, 24). The question arises if the increase in membrane protein glycosylation might be somehow related to this phenomenon. In this connection it appears of interest that ery-

Table 2 Lysine-bound glucose of membrane proteins and hemoglobin of young and old red cell populations. Experimental details are described in the text.

Patient Nr.		GOT activity in hemolysate U/g hemoglobin	nMol Lys-glu/ mg EM protein	Ratio bottom/top fraction	nMol Lys-glu/ mg hemoglobin	Ratio bottom/top fraction
1	top	6,98	1,70	1,76	1,55	1,76
	bott.	2,50	3,00		2,73	
2	top	5,28	1,67	1,48	1,71	1,46
	bott.	3,46	2,47		2,50	
3	top	7,01	1,82	1,64	1,49	1,58
	bott.	2,15	2,99		2,36	
4	top	5,68	1,29	1,79	1,38	1,87
	bott.	1,94	2,31		2,58	

throcytes were observed to loose part of their deformability also upon aging (25, 26). We have therefore studied if the membranes from young and old erythrocyte populations might present different states of glycosylation. In Table 2 the enrichment of aged red cells in the bottom fraction after centrifugation is confirmed by the marked decrease in GOT activity in agreement with (20). Further, Table 2 then indicates that the older cells which were exposed for longer times to glucose in the circulation exhibit a higher (ca. 1.7 fold) amount of lysine-bound glucose residues of membrane proteins and also of hemoglobin than the young cell populations. Similarly, the proportion of HbA_{Ia+b} and HbA_{Ic} was shown to be higher in the fraction of old erythrocytes (27). Thus it appears that the loss in deformability of diabetic (23, 24) and of aged erythrocytes (25, 27) is associated with an increase in non-enzymatic glycosylation not only of hemoglobin but also of the membrane proteins. Whether this implies a causal interrelationship between structural protein modifications due to sugar attachment and changes of the physical properties of red cells remains to be established.

ACKNOWLEDGEMENT

This work was supported by the Fritz-Thyssen-Stiftung, Köln, GFR.

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