Nonenzymatic Glycation of Human Platelet Membrane Proteins in Vitro And in Vivo

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Human platelet membrane proteins (PMP), incubated in vitro in the presence of various concentrations of glucose, undergo nonenzymatic glycation, as evidenced by incorporation of [3-14C]glucose radioactivity into the acid-precipitable fraction. The time course of the reaction is linear for the first hours, and the rate of glycation depends on the glucose concentration in the medium: at a glucose concentration of 80 mmol/L, up to 60 nmol of glucose is bound per milligram of PMP. The ketoaminic nature of the glucose/protein linkages was demonstrated by the finding of 5-hydroxymethylfurfuraldehyde by liquid-chromatographic analysis of acid hydrolysates of PMP. We analyzed PMP from 13 subjects with type I poorly controlled diabetes and from 10 nondiabetics. Nonenzymatic glycation, evaluated as nanomoles of the aldehyde per milligram of protein, was much greater in diabetic patients than in nondiabetics: 1.58 ± 0.70 vs 0.37 ± 0.18 (mean ± SD).

Additional Keyphrases: diabetes, chromatography, liquid, 5-hydroxymethylfurfuraldehyde

Abnormalities in platelet clumping (1), aggregation (1,2), and metabolism (1,3-9) in diabetes mellitus are well known. These altered platelet functions, usually accompanied by microangiopathy, are poorly understood, but a growing body of evidence indicates that nonenzymatic glycation (10) of proteins is one of the most important molecular alterations (11) in the pathogenesis of microangiopathy. Indeed, such a chemical insult—nonenzymatic glycation—is responsible for altering the function of the proteins involved. Glycated hemoglobin has an increased affinity for O2 (12); glycated spectrin may account for reduced erythrocyte deformability in diabetes (13); glycated low-density lipoproteins lose their affinity for the receptor (14), whereas catabolism of glycated high-density lipoproteins is accelerated in experimental animals (15); and the altered physical properties of lens crystallin by glycation may be involved in cataract formation in diabetes (16, 17).

Other cellular abnormalities, such as the increased platelet aggregation and the increased synthesis of thromboxane A in diabetics, have been correlated with hyperglycemia (18, 19), just as the normalization of these abnormalities has been correlated with euglycemia (20).

Further identification of specific proteins exposed to nonenzymatic glycation and elucidation of the subsequent effects of glycation on protein functions are essential for explaining the pathogenesis of long-term diabetic complications in terms of chronic hyperglycemia (21).

The aim of the present study was to test the hypothesis of whether the proteins of human platelet membranes also undergo nonenzymatic glycation in vitro and in vivo, given their continuous exposure to blood glucose and the availability of their lysine and valine residues (22) for condensation with glucose (23).

We present here the first evidence of in vitro and in vivo nonenzymatic glycation of human platelet membrane proteins (PMP).

Materials and Methods

Patients. We studied 13 type I diabetics attending our Metabolic Unit who were in poor metabolic control (mean values for glycated hemoglobin 12.2%, SD 1.7%). They were receiving no therapy other than insulin and had no clinical evidence of renal and hepatic impairment or of plasma lipid alterations. Ten healthy volunteers composed the control group.

After they had fasted for 12 h, we collected blood specimens for PMP preparation, and for determination of glycemia, HbA1, and glycated plasma proteins.

Platelet membrane preparation. Blood was collected with sodium citrate as anticoagulant. The platelets were isolated by two cycles of differential centrifugation (22) and successively freed of residual erythrocytes by repeated differential centrifugation at 1500 × g in 1-min bursts; the platelet preparation was then sedimented by centrifugation at 4000 × g for 15 min and washed three times in citrate-saline solution (sodium citrate, 31.8 g/L, diluted 20-fold with isotonic saline) (22). Leukocyte contamination was less than 1 per 1000 platelets.

We then lysed the platelets by adding 20 μL of Triton X-100 detergent (200 mL/L solution) per milliliter of platelet suspension and checked the completeness of platelet lysis by use of phase-contrast microscopy. To separate the membranes from the platelet extract, we centrifuged at 40 000 × g (20 min, 4°C), then washed the pellet three times and resuspended it in phosphate-buffered saline (phosphate 38 mmol/L, EDTA 0.1 g/L, NaCl 155 mmol/L, pH 7.4). Protein was determined in all samples by the method of Lowry et al. (24).

Usually, analytical recovery of proteins from platelet membrane was about 1 mg per 36 mL of blood.

Incubation. For the in vitro glycation, the PMP (1 g/L) were incubated in the dark and under sterile conditions at 37°C with glucose, 5–80 mmol/L. Trace amounts of [3-3H]glucose (supplied as 10.8 kCi/mol) were included in the incubation mixture, for use in monitoring glucose uptake.

We assayed acid-precipitable radioactivity as described previously (25). Ketoaminic linkages were also detected by liquid chromatography (see below) as 5-hydroxymethylfurfuraldehyde (5-HMF).

We used the results of Day et al. (25) regarding the optimum time course, reaction rate, and incubation conditions for glycation of albumin. Thus, to verify our experi-
mental conditions, we always incubated, in parallel with PMP, human albumin (Calbiochem-Behring, La Jolla, CA), 1 g/L, with glucose, 20 mmol/L.

5-HMF assay by liquid chromatography. We measured nonenzymatic glycation of PMP and plasma proteins from diabetic patients and controls by measuring the 5-HMF liberated after acid hydrolysis. 5-HMF has been widely measured colorimetrically by its reaction with thiobarbituric acid (26). Now it can be measured with much more accuracy and sensitivity by liquid chromatography (27, 28). Briefly, the procedure is as follows. An aliquot of each sample is incubated with NaBH₄ at 25°C for 1 h to reduce ketoamino linkages. Both reduced and nonreduced aliquots of the samples are hydrolyzed with 1 mol/L oxalic acid (0.5 mL per milliliter of sample) for 2 h in a boiling water bath. After cooling, the samples are precipitated with trichloroacetic acid and the supernates are filtered (Millipore filters, 0.22-μm av pore-size) before the chromatographic analysis. We used the Model ALC 206 isocratic chromatographic system and the 30 × 3.9 cm Bondapak C₁₈ (10 μm) reversed-phase liquid-chromatographic column (Waters Associates, Milford, MA). For the elution, sodium acetate (5 g/L, pH 4.3) is used at a flow rate of 2 mL/min. Absorbance is recorded at 280 nm and the detector sensitivity is set at 0.01-A full scale. We injected 100 μL of sample into the chromatograph. For the standard, we used an aqueous solution of 5-HMF (Sigma Chemical Co., St. Louis, MO).

To detect substances possibly interfering with the 5-HMF peak, we always recorded the ratio of the peak heights A₂₈₀/A₂₅₄, which was 4.4 in the absence of interferents. Because the relation between peak height and concentration of 5-HMF standards is linear, the calculation of 5-HMF formed in the samples was based on peak heights and expressed as nanomoles per milligram of protein.

The 5-HMF in the reduced samples was always subtracted from that in the nonreduced samples. The 5-HMF liberated per milligram of protein was constant for the same sample at different protein concentrations between 0.8 and 10 g/L. The mean CV for the within-assay precision, tested by measuring 5-HMF of the same serum pool at six different concentrations, was 6.8% (n = 12). The mean between-assay reproducibility, calculated on the basis of six different determinations of the same pool, was 11.6% for the entire procedure.

HbA₁ was measured with a kit (Bio-Rad Labs., Richmond, CA 94804), plasma glucose with a Beckman analyzer.

Results

In vitro studies. PMP incubated with various concentrations of glucose (5–80 mmol/L) and trace amounts of [3-³H]glucose showed accumulation of radioactivity in the acid-precipitable fraction. The glucose incorporation measured through a six-day period (i.e., the half-life of platelets) was greatest within the first 24 h, then it plateaued (Figure 1a). Analysis of the first 24 h in more detail showed that most of the glucose incorporation occurred in the first 9 h (Figure 1b). In contrast, the time-dependent glucose incorporation into human albumin in the same experimental conditions was linear through the sixth day (Figure 2).

As depicted in Figure 3, glucose incorporation in PMP during a 24-h period was directly linearly correlated with the glucose concentration in the medium. Based on the approximate specific activity of [3-³H]glucose in the incubation mixture, incorporated radioactivity averaged 60 nmol of glucose per milligram of PMP at the maximum glucose concentration (80 mmol/L).

To verify whether the PMP-glucose binding was ketoaminc, we incubated PMP with glucose (80 mmol/L), removed the unbound glucose after various incubation times (0, 6, 24, 48, and 96 h), and hydrolyzed the remaining contents. Chromatographic analysis (Figure 4) of hydroly-sates showed that 5-HMF increased with the incubation time: 5-HMF was in fact 0.5 nmol/mg of PMP at time zero, increasing to 7.4, 16.4, 19.0, and 19.2 nmol/mg at 6, 24, 48, and 96 h, respectively. Thus, the plateau in the ketoamino linkage formation was attained in approximately 48 h (Figure 5).

In vivo studies. The results of the in vivo evaluations are summarized in Table 1, together with the data regarding metabolic control. PMP from diabetic subjects showed four-
fold greater nonenzymatic glycation than did that from control subjects. Similarly, the mean value of 5-HMF from plasma proteins of diabetics was twice the value in control subjects.

**Discussion**

The present study shows that PMP incubated with glucose undergo nonenzymatic glycation in vitro. Glycation rate is a function of glucose concentration in the medium, at least in the range explored (5–80 mmol/L). Under the experimental conditions of this study, particularly regarding protein concentration (1 g/L), most of the glucose was incorporated within the first 24 h. This was surprising: we had expected instead a slower linear increase through the six-day period, as was the case for albumin. On the other hand, because the linearity of the glucose incorporation into albumin through six days of incubation was consistent with the data from the literature (25) we could exclude that the unexpected glycation reaction for PMP was related to experimental variables such as temperature variation, bacterial contamination, etc.

The ketoaminic nature of PMP-glucose linkage was demonstrated by the chromatographic measurement of 5-HMF in acid hydrolysates of PMP after incubation with glucose. Even here the reaction was complete in the first 48 h, though slightly later than the incorporation of radiolabeled glucose. Measurement of radioactive glucose uptake accounts also for Schiff base formation, whereas measurement of 5-HMF accounts only for hydrolyzed ketoaminic linkages.

Nnonenzymatic glycation of PMP also takes place in vivo. In fact, PMP from diabetic patients showed much greater glycation than PMP from nondiabetics. PMP glycation exceeded the glycation of plasma proteins in the individual subjects, either diabetic or nondiabetic, presumably because the PMP have more exposed residues available for glucose binding than do other proteins.

The conditions for glycation of PMP in vivo obviously differ from those occurring in vitro: in vivo, the PMP are part of intact and viable cells, the glucose concentrations are lower (even when the diabetics develop frank hyperglycemia), and the glucose/protein ratio is different.

If the glycation kinetics were the same in vitro and in...
vivo, a 24- to 48-h period of hyperglycemia would be enough to produce the maximum of structural modification. From this, we speculate that even very short periods of hyperglycemia are dangerous to diabetic patients, should platelets be subject to a causal relationship between glycation and altered function.

These results are particularly important in view of the key role played by the membrane in platelet physiology, with special regard to platelet–platelet interaction and platelet adhesiveness to endothelium. Future investigations will be aimed at ascertaining whether a causal relation exists between membrane nonenzymatic glycation and platelet aggregation.

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References