Regulation of Hemoglobin A_{Ic} Formation in Human Erythrocytes In Vitro

EFFECTS OF PHYSIOLOGIC FACTORS OTHER THAN GLUCOSE

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ABSTRACT The formation of hemoglobin A_{Ic} was studied in intact human erythrocytes in vitro. Satisfactory methods were developed for maintaining erythrocytes under physiologic conditions for >8 d with <10% hemolysis. Hemoglobin A_{Ic} levels were determined chromatographically on erythrocyte hemolysates after removal of reversible components by incubation for 6 h at 37°C. Hemoglobin A_{Ic} concentration was found to increase linearly with time during 8 d of incubation. The rate of formation of hemoglobin A_{Ic} increased linearly as glucose concentration was increased from 40 to 1,000 mg/dl. Deoxyhemoglobin was glycosylated twice as rapidly as oxyhemoglobin. The rate of hemoglobin A_{Ic} formation was further increased by elevated 2,3-diphosphoglycerate levels, an effect that was most marked with deoxyhemoglobin. We conclude that the nonenzymatic glycosylation of hemoglobin is influenced by factors other than glucose, including oxygen tension and 2,3-diphosphoglycerate levels.

INTRODUCTION

Hemoglobin A_{Ic} is a minor component of the human erythrocyte that constitutes 5% of the total hemoglobin in normal adults and up to 15% in patients with diabetes mellitus (1). It is formed by the nonenzymatic condensation of glucose with the amino-terminal valine residues of the β-chains of hemoglobin A (2, 3). Glucose binds reversibly to hemoglobin as an aldimine (Schiff base), and this adduct then undergoes an Amadori rearrangement to form a stable ketoamine (1-deoxy, 1-aminofructose) (3).

The levels of hemoglobin A_{Ic} in diabetic patients correlate with blood glucose levels (4). It is assumed that the mean glucose concentration is the primary determinant of the relatively slow rate of hemoglobin A_{Ic} formation (5), thus making a single hemoglobin A_{Ic} determination a useful index of blood glucose control in diabetics over the preceding several weeks. Studies with isolated erythrocytes at 4°C have confirmed a linear relationship between glucose concentration and hemoglobin A_{Ic} formation (6), but long-term in vitro experiments with hemoglobin in its native state in erythrocytes at 37°C have not been reported. For this reason, we have developed methods for incubating intact human erythrocytes for >8 d. We have defined the relationship between extraerythrocyte glucose concentration and hemoglobin glycosylation and also have found that physiologic factors other than glucose influence the rate of glycosylation.

METHODS

Erythrocyte incubation methods. After obtaining informed consent, ~30 cm^3 of blood was withdrawn from an antecubital vein of a healthy adult volunteer and placed in a sterile syringe containing heparin (14 U/cm^3). The blood was transferred to a sterile centrifuge tube on ice, a small aliquot was removed for base-line hemoglobin A_{Ic} determination, and the remainder was centrifuged for 10 min at 1,000 *g*. After removal of plasma and the buffy coat, the erythrocytes were washed twice by suspending them in cold Dulbecco's phosphate-buffered saline and recentrifuging for 5 min at 1,000 *g*. The final pellet of washed erythrocytes was pipetted into incubation medium at a concentration of 1% (vol/vol), and the resulting suspension was pipetted into 100-mm petri dishes (10 ml/dish).

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The incubation medium consisted of 90% Earle's balanced salt solution (7) and 10% bovine serum (Gibco Laboratories, Grand Island Biological Co., Grand Island, N. Y.), plus the indicated concentrations of glucose and other additives. Phenol red was excluded from the medium to avoid interference with spectrophotometric studies. The pH was maintained at 7.4 by incubation in an atmosphere containing 5% CO_2.

Determination of hemolysis. After various periods of incubation, the erythrocytes were suspended in the medium by swirling the dish and then transferred to a graduated centrifuge tube. The volume was recorded, and the suspension was centrifuged for 10 min at 1,000 *g*. A 5-ml aliquot of the supernatant was transferred to a separate tube and centrifuged for 60 min at 45,000 *g*. The supernatant was used for determination of free hemoglobin. The erythrocyte pellet obtained from the first centrifugation was hemolyzed by adding distilled water to the level of the original volume. The hemolysate was clarified by centrifuging for 15 min at 2,000 *g*. The resulting supernatant was diluted 10-fold with water and used for determination of erythrocyte hemoglobin. The absorbances of the medium supernatant and the erythrocyte hemolysate were determined at both 525 and 472 nm (isobestic wavelengths for oxy and methemoglobin), and hemolysis was calculated by the following formula:

$$\text{Percent hemolysis} = (\text{Medium OD})/(\text{Medium OD} + \text{Erythrocyte OD}) \times 100.$$

Determination of hemoglobin A_{Ic}. The erythrocytes were suspended by swirling the culture dishes and then were transferred to centrifuge tubes. After centrifugation for 10 min at 2,000 *g*, the erythrocyte pellets were washed twice with saline and hemolyzed in 1 ml of water. Lipid components were removed by vortexing with 1 ml of toluene for 1 min. The resulting emulsion was centrifuged for 10 min at 2,000 *g*. The aqueous, infranatant layer was removed and stored in liquid nitrogen. Before analysis, the samples were incubated for 6 h at 37°C to eliminate the reversible aldimine form of hemoglobin A_{Ic}. The irreversible ketoamine form was then determined by high pressure chromatography on Biorex 70 ion exchange resin as previously described (8).

Determination of 2,3-diphosphoglycerate (DPG).[1] DPG was determined with Sigma Kit 665-PA (Sigma Chemical Co., St. Louis, MO) modified to decrease sample size. In brief, inorganic phosphate was measured by the colorimetric method of Fiske and Subbarow (9) following its specific release from DPG by the action of diphosphoglycerate phosphatase in the presence of 2-phosphoglycolic acid.

RESULTS

When 10 ml of a suspension of erythrocytes with a hematocrit of 1.0% was incubated in a 100-mm petri dish, the erythrocytes settled to the bottom of the dish and formed a layer one cell thick. After 8 d, <10% of the starting hemoglobin was released to the medium by hemolysis (measured at both 472 and 525 nm). Spectrophotometric analysis of erythrocyte hemoglobin showed no significant methemoglobin formation.

When erythrocytes were incubated in medium with relatively high glucose concentrations, there was a rapid rise in hemoglobin A_{Ic} (aldimine plus ketoamine). This was determined by measuring hemoglobin A_{Ic} on samples that had been stored for only short periods of time in liquid nitrogen and then analyzed immediately to prevent dissociation of the aldimine. At a glucose concentration of 1,000 mg/dl, 5% of the total hemoglobin A was converted to hemoglobin A_{Ic} in 24 h. This was largely attributable to formation of the aldimine (Schiff base). Levels of the irreversible, ketoamine form of hemoglobin A_{Ic}, measured in erythrocyte hemolysates that had been preincubated for 6 h at 37°C, increased at a much lower, constant rate during the 8 d in vitro. In all subsequent experiments, hemoglobin A_{Ic} levels were only determined on samples that had been preincubated to eliminate the aldimine. Under these conditions, glycosylation proceeded at a linear rate for 8 d at all glucose concentrations between 40 and 1,000 mg/dl.

The rate of ketoamine formation was directly proportional to the glucose concentration in the medium (Fig. 1) and is described by the following formula:

$$\text{Rate of hemoglobin } A_{Ic} \text{ formation (\%/24 h)} = (3.52 \times 10^{-4})\ (\text{mg glucose/dl}) + (4.09 \times 10^{-2}).$$

For a mean glucose concentration of 100 mg/dl, the rate of ketoamine A_{Ic} formation in the incubated erythrocytes was 0.076%/24 h, which is identical to the rate determined by following the in vivo incorporation of ^{59}Fe into hemoglobin A_{Ic} in a normal human (10).

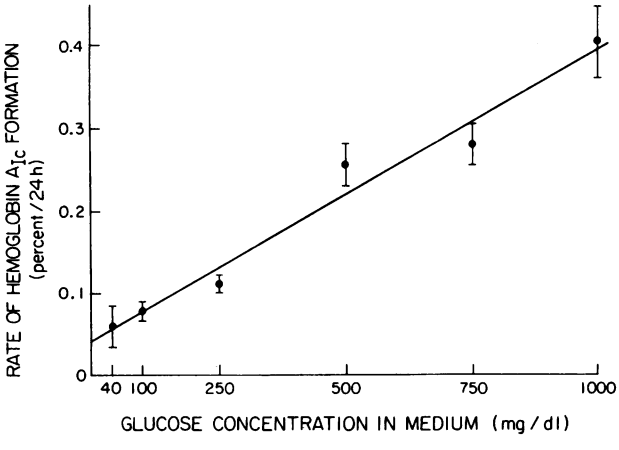

FIGURE 1 The effect of glucose concentration on the rate of hemoglobin A_{Ic} formation. Erythrocytes were incubated at a fixed glucose concentration and samples were analyzed for hemoglobin A_{Ic} on days 1, 2, 3, 4, 6, and 8. All samples were analyzed on the same day (coefficient of variation = 3%). The rate of hemoglobin A_{Ic} formation was determined by linear regression. Each point represents the mean±SE for three experiments at the indicated glucose concentration. The actual hemoglobin A_{Ic} level on day 0 was 5.8±0.3.

[1] *Abbreviation used in this paper*: DPG, 2,3-diphosphoglycerate.

The pH of the medium was altered by changing the $NaHCO_3$ concentration (replacing it with NaCl) and keeping the atmospheric CO_2 constant at 5%. Changes in pH between 7.0 and 8.0 did not alter hemoglobin A_{Ic} formation (data not shown). Medium pH beyond the range of 7.0 to 8.0 led to rapid hemolysis.

When erythrocytes were incubated under anaerobic conditions (95% N_2, 5% CO_2) the rate of hemoglobin A_{Ic} formation increased twofold over the rate with room air plus 5% CO_2 (Table I). Levels of O_2 that were calculated to partially saturate hemoglobin resulted in intermediate rates of hemoglobin A_{Ic} formation. Addition of 0.3% carbon monoxide to anaerobic cultures restored hemoglobin A_{Ic} formation to the aerobic rate. Thus, the increased glycosylation of hemoglobin when O_2 partial pressure is decreased must result from the change in hemoglobin conformation and not from some other metabolic effect of hypoxia on the erythrocytes.

To achieve and maintain elevated DPG levels, methods developed for restoring DPG in banked blood were adapted for 37°C culture conditions (11). Supplementation of the usual incubation medium with 5 mM NaH_2PO_4, 2.5 mM inosine, and 2.5 mM sodium pyruvate resulted in sustained increases in DPG to approximately twice normal. The levels of erythrocyte DPG measured after 8 d in culture are shown in Table II. The presence of O_2 had no effect on DPG levels. The low DPG concentrations are similar to in vivo levels in diabetic ketoacidosis (12), and the high concentrations are similar to clinical states with systemic hypoxia (13).

TABLE I
Effect of P_{O_2} on Hemoglobin A_{Ic} Formation

Experimental conditions	Rate of Hb A_{Ic} formation
	%/24 h
Room air (20% O_2)	0.38±0.05
3.5% O_2	0.52
Anaerobic	0.73±0.05*
Anaerobic + 0.3% CO	0.38

Erythrocytes were incubated in a medium containing 1,000 mg/dl glucose in an atmosphere of 5% CO_2 and the indicated concentrations of O_2 or CO. Samples were analyzed for hemoglobin A_{Ic} on days, 1, 2, 3, 4, 6, and 8, and the rate of hemoglobin A_{Ic} formation was determined by linear regression. Data represent mean±SE for three experiments or the mean of two experiments.

* Significantly greater than the rate in room air, $P < 0.025$, unpaired t test.

TABLE II
Effect of DPG on Hemoglobin A_{Ic} Formation

Experimental conditions	DPG concentration	Rate of hemoglobin A_{Ic} formation
	μmol/ml erythrocytes	*%/24 h*
Aerobic, low DPG	0.68±0.38	0.36±0.04
Aerobic, high DPG	8.90±3.27	0.47±0.04*
Anaerobic, low DPG	0.80±0.25	0.61±0.03*
Anaerobic, high DPG	8.78±1.70	0.94±0.01*

Erythrocytes were incubated in medium containing 1,000 mg/dl glucose in an atmosphere of 5% CO_2 and either 95% room air or 95% N_2. Samples designated "high DPG" were supplemented with phosphate, inosine, and pyruvate as described under Methods. Samples designated "low DPG" were unsupplemented. DPG concentration in the cells was determined after 8 d in culture. Hemoglobin A_{Ic} was measured on days 1, 2, 3, 4, 6, and 8, and the rate of formation was determined by linear regression. Data represent mean±SE for three experiments.

* Significantly greater than aerobic, low DPG, $P < 0.005$, unpaired t test.

Under aerobic conditions, the rate of hemoglobin A_{Ic} formation is 30% higher in "high DPG" cultures than in "low DPG" cultures (Table II). Under anaerobic conditions, elevated DPG leads to a 50% increase in the rate of hemoglobin A_{Ic} formation. Thus, the effect of DPG on glycosylation is greatest when hemoglobin is in the deoxy-conformation.

DISCUSSION

Previous studies on hemoglobin A_{Ic} formation in vitro in intact erythrocytes at physiologic temperatures have been limited to 24–48 h because of extensive hemolysis (6, 14). We have found that supplementation of a simple balanced salt solution with 10% bovine serum allows the incubation of washed human erythrocytes at 37°C for >8 d with minimal hemolysis. The experimental model appears to be valid for studies on glycosylation, since hemoglobin is maintained in its native state and hemoglobin A_{Ic} formation occurs at a rate that is similar to the estimated in vivo rate. Under these conditions, we have observed a linear relationship between hemoglobin A_{Ic} formation and glucose concentration in the medium.

It was not possible to study hemoglobin glycosylation at glucose concentrations <40 mg/dl because of excessive hemolysis. As noted in Fig. 1, however, extrapolation from data at higher glucose concentrations indicates a finite rate of hemoglobin A_{Ic} formation in the absence of glucose in the medium (0.041%/24 h).

This could potentially result from: (*a*) more efficient uptake of glucose by erythrocytes at low glucose concentrations, (*b*) sequestration of glucose at the hemoglobin binding site, or (*c*) heterogeneity of the hemoglobin A_{Ic} peak. The specific mechanism has not been identified.

It has been assumed that the nonenzymatic glycosylation of hemoglobin is dependent only on glucose concentration, and thus that hemoglobin A_{Ic} levels are determined by the mean plasma glucose concentration and the erythrocyte life span (10). By studying intact erythrocytes in vitro, however, we have found that other physiologic factors have a potent influence on the rate of hemoglobin A_{Ic} formation. At a constant glucose concentration, deoxyhemoglobin is glycosylated at a twofold higher rate than oxyhemoglobin. Thus, hemoglobin A_{Ic} levels may lead to an overestimation of mean glucose concentrations in patients who have pulmonary disease with systemic hypoxia or vascular disease with extensive areas of venous stasis and local erythrocyte hypoxia. The mechanism of the enhanced glycosylation of deoxyhemoglobin is unknown. There is precedent, however, for increased reactivity of the amino-termini of deoxyhemoglobin, since the binding of both CO_2 and cyanate is enhanced in the deoxy state (15, 16).

The rate of formation of hemoglobin A_{Ic} also is influenced by the level of DPG. As the DPG concentration rises, hemoglobin glycosylation increases. In the extreme, deoxyhemoglobin in the presence of a high physiologic DPG level is glycosylated at a threefold greater rate than oxyhemoglobin in the presence of a low physiologic DPG level.

The mechanism of the DPG effect is unclear. Perhaps DPG either decreases the effective positive charge on the amino terminus, thus increasing its reactivity with glucose, or causes a conformational shift that results in improved steric factors. Alternatively, it is possible that we are not measuring the formation of authentic hemoglobin A_{Ic}, but rather the formation of an uncharacterized hemoglobin adduct that happens to cochromatograph with hemoglobin A_{Ic}. This seems unlikely, since measurement of glycolytic intermediates in erythrocytes treated with inosine, pyruvate, and phosphate has shown no changes in the concentrations of compounds that contain potentially reactive carbonyl groups (17). Furthermore, a previous study of multiple glycolytic intermediates showed that when hemoglobin adducts are formed, they always cochromatograph with hemoglobin A_{Ib} (18). It is unlikely that pyruvate itself is reacting with hemoglobin A, since the addition of 2.5 mM sodium pyruvate alone to the incubation medium did not result in increased hemoglobin A_{Ic} formation (data not shown).

It should be noted that we have not actually determined that DPG and the anaerobic state increase the reaction rate between glucose and the hemoglobin β-1 valine. Since we are measuring the stable ketoamine product, it is also possible that these interventions increase the rate of the Amadori rearrangement that converts the Schiff base to the ketoamine.

The effects of O_2 and DPG on hemoglobin glycosylation may explain some of the variability in hemoglobin A_{Ic} levels observed in different diabetic patients with similar blood glucose levels or in the same patient at different times. In addition, the concept of modification of the rate of nonenzymatic glycosylation may also be relevant to glycosylation of other proteins at their amino-termini or lysine ϵ-amino groups. Similar effects by other physiologic factors could lead to differences in protein glycosylation and possibly in the severity of diabetic complications.

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