Anion Transport as Related to Hemoglobin A1c in Erythrocytes of Diabetic Children

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We determined chloride and bicarbonate transport \( [J_d \text{ and } J_b] \) in erythrocyte membrane samples from diabetic and non-diabetic children. CO2 transport capacity of the blood was not impaired in diabetics and consequently does not endanger the compensatory hyperventilation after ketoacidosis.

Additional Keyphrases: blood gases · electrolytes · diabetes · CO2 transport capacity of blood · ketoacidosis · glycated proteins · capnophonn

Ketoacidosis, a well-known complication in hyperglycemic diabetic patients, is characterized by a metabolic acidosis accompanied by a compensatory hyperventilation. The hyperventilation creates a steeper gradient for the carbon dioxide tension between tissues and lung alveoli, so that more acid equivalents in the form of CO2 can be expired. A maximum exploitation of the increased CO2 gradient as the driving force for an increased CO2 expiration depends heavily upon an intact function of the so-called Jacobs–Stewart cycle (1). This cycle describes the diffusion of CO2 from the tissue into the blood, the enzymatically governed conversion of CO2 within the erythrocyte into bicarbonate, and the subsequent transport of bicarbonate ions into the plasma in exchange for plasma chloride ions (the Hamburger or chloride–bicarbonate shift). As the blood passes the lung capillary, the reaction steps run backwards, and the CO2 is given off to the alveolar air. The rate-limiting step in the Jacobs–Stewart cycle is the Hamburger shift (2), which is mediated by an integral membrane protein called "capnophorn" (or "band 3"). The results of previous studies (3–5) suggest that any condition leading to hyperventilation, such as exercise, anemia, and acidosis, may be aggravated by even a partial inhibition of the Hamburger shift.

The goal of the present study was to elucidate whether poor glycem control as assessed by measurement of hemoglobin A1c, which also reflects the degree of glycation of the membrane proteins (6), affects the anion-transport system in erythrocytes, owing to glycation of a lysine group that is essential for intact transport function (7).

Materials and Methods

Subjects. Twelve children (six boys, six girls) with insulin-dependent diabetes mellitus attended the outpatient clinic for diabetic care each month. Ten control children (four girls, six boys) were non-diabetic. After informed consent, we collected 20 mL of heparinized whole blood for determination of actual blood glucose concentration, HbA1c, and the bicarbonate and chloride exchange across the erythrocyte membrane.

The ages of the diabetic children ranged from 11 to 19 y (mean = 15 y) and the mean duration of their diabetes was 4.5 y (range, one to 15 y). All were treated with a combination of short- and long-acting insulin, the mean dosage being 0.95 USP units per kilogram of body weight per 24 h (range 0.65–1.35 units/kg). Nine were treated with a multiple injection regimen, with use of an insulin-injection pen, while three others had insulin twice daily. None of the children had manifestations of late diabetes. In all of the diabetic children the actual HbA1c concentration had been consistent within 1% during the last six months (mean of six determinations).

The ages of the non-diabetic controls ranged from nine to 16 y (mean = 13 y). They were of normal body weight (8) and none had diabetes in their family. All had values for HbA1c and blood glucose within the normal reference intervals.

Determination of blood glucose concentration and HbA1c. Blood glucose concentration was measured by a glucose dehydrogenase (EC 1.1.1.47) method, in a continuous-flow instrument (AutoAnalyzer; Technicon, Tarrytown, NY). The substance fraction of HbA1c was determined by isoelectric focusing on a slab of polyacrylamide gel (Ampholine PAG plate, cat. no. 1804–131; LKB-Products, Bromma, Sweden) followed by quantification of hemoglobins by spectrophotometry. By this technique the aldime adduct, which we designate HbA1d, is well separated from the ketoamine HbA1c (9). Non-incubated hemolysates with a substance concentration of hemoglobin (Fe) of 2 to 3 mmol/L were prepared from 0.5 mL of washed and packed erythrocytes lysed in 3.5 mL of distilled water. The samples, stabilized by bubbling with carbon monoxide, were stored at −20 °C for about 48 h until analysis was performed.

Our reference interval for HbA1c is 4.0–6.5% (mean 5.3%) of total hemoglobin. The interassay coefficient of variation for HbA1c samples in the reference interval is 0.043. In the range from 8 to 14% this analytical coefficient of variation is 0.038.

Determination of anion exchange in erythrocytes. Details of the procedures have been described previously (10, 11), so that only a brief description of the standard procedure for each blood sample is given here. Heparinized blood, with buffy coat removed after centrifugation, was washed once, titrated to pH 7.4 at 38 °C with CO2 or a 100 mmol/L solution of KOH, and washed three more times in a medium containing, per liter, 110 mmol of KCl, 25 mmol of KHCO3, 1 mmol of acetazolamide, and 30 mmol of sucrose.

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composition ensures iso-osmolarity and physiological concentrations of the anions concerned. Between the two first washes, the cells were incubated at 38 °C for 30 min, which exceeds the time required for equilibration between the intracellular and extracellular phase of the carbonic anhydrase inhibitor, acetazolamide (Diamox). After the last wash, the cells were resuspended to give a hematocrit of about 50% in the medium, and the suspension was divided into two portions, one for loading the erythrocytes with $^{36}$Cl$^-$ (AEK, Risø, Denmark) and one for loading the cells with $[^{14}C]$HCO$_3^-$ (Amersham International plc, Amersham, Buckinghamshire, U.K.). The radiolabeled erythrocytes were packed in appropriate tubes (10) for determination of (a) the distribution of radioactive isotope between the cell water phase and the medium, (b) of the cell water volume (by drying the cell sample to constant weight), and (c) for efflux experiments with the rapid continuous-flow tube method, which is well suited for measuring transport processes that last for only a fraction of a second (cf. Figure 1).

The rate of anion exchange ($k$, s$^{-1}$) under equilibrium conditions, as determined as the rate of the unidirectional efflux of the radioactive anion, is related to the apparent permeability ($P_{app}$, cm/s) and the unidirectional flux ($J_{uni}$, mol/cm$^2$·s) by:

$$J_{uni} = k \cdot (V/A) \cdot C_{in} = P_{app} \cdot C_{in}$$  

where $V/A$ (cm$^3$) is the ratio between the cell water volume and the membrane area, which is assumed to be constant (1.42·10$^{-6}$ cm$^2$ per cell), and $C_{in}$ is the intracellular anion concentration (mol/cm$^3$). In all experiments the efflux rate could be described by a monoexponential function where $-k$ equals the exponent that represents the slope of the efflux curve as depicted in a semilogarithmic plot shown in Figure 1.

**Results**

Figure 1, a semilogarithmic plot, illustrates the rates of efflux of $^{36}$Cl$^-$ and $[^{14}C]$HCO$_3^-$, determined in the same specimen. Duplicate determinations of the efflux rate of both anions were done, when possible for all blood samples, both from diabetic and non-diabetic children. The results are summarized in Table 1, which also includes a control experiment on anion transport in a blood sample from a non-diabetic adult donor. Table 1 shows that the chloride and bicarbonate exchange fluxes are similar in erythrocytes from diabetic and non-diabetic children, and from the adult. Furthermore, there was a highly statistical significant difference ($P < 0.001$) between the diabetic and control groups in glucose concentration and HbA$_{1c}$ values, which also reflect the different degree of glycation of the membrane proteins in the two populations.

**Discussion**

Hyperglycemia nonspecifically increases non-enzymatic glycation of proteins and alters a series of physiological processes (12). There is strong evidence that inactivation of ribonuclease alpha (EC 3.1.28.2) and erythrocyte superoxide dismutase (EC 1.15.1.1) activity is connected with glycation of lysine epsilon-amino groups (13, 14), and it has been predicted that glycation of such amino groups may also inactivate other enzymes, such as decarboxylases and aldolases, the activity of which depends on "unsubstituted active site lysine amino groups" (12).

Because the erythrocyte membrane proteins, including the integral transport proteins, appear as susceptible to glycation as other protein sites (6), the function of the proteins may be altered in diabetic patients.

Previous studies have shown that the facilitated diffusion of glucose is not changed in erythrocyte membranes of diabetic subjects and that insulin has no effect on glucose transport in erythrocytes of non-diabetics (15, 16). Jennings et al. (17) showed that the active sodium efflux is significantly reduced in erythrocytes from diabetics, and they also showed that the Na,K-transporting ATPase (EC 3.6.1.37) activity in control cells is insensitive to an increased glucose concentration and to insulin. Baldini et al. (18), on the other hand, demonstrated that insulin stimulates active Na$^+$ transport by enhancing the availability of pumping sites.

We here primarily address the question whether a pathophysiological glycation of the erythrocyte anion-transport system may affect the CO$_2$-transport capacity of the blood.

Under physiological conditions at rest with a cardiac output of 4 to 5 L of blood per minute, each liter of blood carries about 2 mmol of CO$_2$, which is produced in the tissues, to the lungs. Under heavy muscle exercise, 1 L of blood transports about 6 mmol of CO$_2$ per minute. As much as 80% of the CO$_2$ taken up in the tissue capillary is

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**Table 1. Chloride and Bicarbonate Transport at 38 °C, pH 7.4, and Concentration of Glucose and HbA$_{1c}$ in Erythrocytes from Diabetic Children and Non-Diabetic Controls**

<table>
<thead>
<tr>
<th></th>
<th>$J_{cl}$ (mol/cm$^2$·s)</th>
<th>$J_{bic}$ (mol/cm$^2$·s)</th>
<th>Blood glucose (mmol/L)</th>
<th>HbA$_{1c}$ (%)</th>
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<tr>
<td>Mean</td>
<td>26.1</td>
<td>7.6</td>
<td>10.1*</td>
<td>11.1*</td>
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<td>±3.7</td>
<td>±0.9</td>
<td>±5.9</td>
<td>±2.5</td>
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<tr>
<td>n</td>
<td>24</td>
<td>19</td>
<td>12</td>
<td>12</td>
</tr>
<tr>
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</tr>
<tr>
<td>Mean</td>
<td>30.6</td>
<td>7.3</td>
<td>4.6</td>
<td>5.4</td>
</tr>
<tr>
<td>SD</td>
<td>±4.6</td>
<td>±1.0</td>
<td>±0.5</td>
<td>±0.9</td>
</tr>
<tr>
<td>n</td>
<td>16</td>
<td>20</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td><strong>Adult</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Range</td>
<td>30.1–31.8</td>
<td>7.7–9.6</td>
<td>not detd.</td>
<td>not detd.</td>
</tr>
</tbody>
</table>

The groups under study consisted of 12 diabetic and 10 non-diabetic children. For comparison, the average and the range of values of duplicate experiments performed with a blood sample from a non-diabetic adult are also shown. The asterisks denote values that are significantly different from the respective control values ($P < 0.001$).
converted to bicarbonate, mainly inside the erythrocyte, whose content of carbonate dehydratase (EC 4.2.1.1) implies a >10000-fold increase of the rate of hydration of CO₂ to carbonic acid. H₂CO₃ is "instantaneously" dissociated into H⁺, which is buffered primarily by hemoglobin, and HCO₃⁻, of which about three quarters is exchanged with plasma Cl⁻ by means of the chloride–bicarbonate or Hamburger shift. This mechanism results in maximum exploitation of the CO₂-transport capacity of the blood with a minimum partial pressure gradient for CO₂ between the tissues and the arterial blood as it enters the capillaries.

In vitro studies have shown that one lysine group in the 17K segment of capnophorin is crucial for an intact function, because anion transport is inhibited if the group is methylated or binds the specific anion-transport inhibitor 4,4'-diisothiocyanato-2,2'-disulfonate [for a review, see Knauf (15)]. Because nearly all of the membrane proteins can be glycated in an approximate proportion to their relative amounts in the membrane, and because lysine groups in the proteins appear particularly susceptible to non-enzymatic glycation (6), high glucose concentrations in the blood might change the properties of the erythrocyte anion-transport system. However, the present results obtained with erythrocytes from diabetic children with abnormally high blood-glucose concentrations during six months or more, as assessed by the proportions of HbA₁c, show that minor inhibition of chloride and bicarbonate transport. It might be argued that we have no evidence for any glycation of the lysine group of capnophorin that is essential for the transport function. However, the question raised here was whether a patho-physiological condition, defined by the value for HbA₁c, that has an effect on the function of other proteins, also affects this particular transport function of the erythrocyte membrane.

We thus conclude from the present study that glycation of capnophorin in the erythrocyte membrane does not substantially affect the anion transport. Because no inhibition of the erythrocyte anion-transport system appears in insulin-dependent diabetes mellitus patients with great protein glycation, we anticipate that hypertentilation after the ketoacidotic state, or simply the state during and after physical performance, is not aggravated by a glycation-induced prolongation of the rate-limiting step in the transport of CO₂ by the blood.

References


