Transmembrane Electron Transfer in Diabetic Nephropathy

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OBJECTIVE — Erythrocytes (red blood cells [RBCs]) reduce extracellular ferricyanide by transmembrane transfer of reducing equivalents involving ascorbate recycling.

RESEARCH DESIGN AND METHODS — Because ascorbate regeneration is glutathione (GSH) dependent and cells may be depleted of GSH in diabetes, we measured RBC GSH, plasma sulfhydryl (SH) groups, and RBC-mediated ferricyanide reduction in 30 type 1 diabetic patients (age 34 ± 10 years, disease duration 20 ± 8 years; no complications, n = 10; retinopathy, n = 10; nephropathy, n = 10), their 36 siblings (age 39 ± 13 years), and matched healthy volunteers.

RESULTS — Fasting plasma glucose was 15 ± 7 mmol/l (vs. 5 ± 1 in control subjects, P < 0.001), HbaA1c 8.4 ± 1.5% (vs. 5.4 ± 0.3, P < 0.001), GSH 0.76 ± 0.12 mg/ml packed RBCs (vs. 0.88 ± 0.18, P < 0.01), SH groups 401 ± 72 µmol/l (vs. 444 ± 56, P < 0.05), and ferricyanide generation 15 ± 5 µmol/ml RBC per h (vs. 13 ± 5, NS). In comparison with 10 normoalbuminuric diabetic subjects with retinopathy, 10 patients with diabetic nephropathy had similar fasting plasma glucose, HbaA1c, and SH groups; lower RBC GSH (0.73 ± 0.08 vs. 0.85 ± 0.11, P < 0.05); and higher ferricyanide generation (18 ± 4 vs. 14 ± 5, P < 0.05). The 10 patients without complications differed from the 10 healthy volunteers in glycemic control and RBC GSH. RBC electron transfer correlated with plasma lactate (r = 0.8, P = 0.01) only in the uncomplicated group. No difference was detected between siblings and healthy control subjects or between siblings of subjects in the nephropathy and retinopathy groups. Among diabetic patients, the rate of ferricyanide generation was associated with urinary albumin excretion, plasma creatinine, and SH groups (multiple r = 0.6, P < 0.01).

CONCLUSIONS — Transmembrane electron transfer is selectively increased in diabetic nephropathy, where RBC GSH is also depleted. The abnormality is peculiar to the nephropa-thy group and not contributed by familial or hereditary components because the electron flow was normal in siblings. The close relationship between cytosolic NADH and RBC electron transfer observed in diabetic patients without complications seems to be lost in the microangiopathic patients. Whereas patients with retinopathy alone still had normal activity of the RBC-reducing system, patients with nephropathy showed significantly increased activity, unrelated to metabolic parameters or plasma lactate concentration and correlated with renal function parameters and plasma thiols.

Diabetes Care 23:994-999, 2000

Human erythrocytes reduce extracellular ferricyanide, [Fe(CN)6]3−, to ferrocyanide, [Fe(CN)6]4−, in a reaction that involves the transmembrane transfer of reducing equivalents (1). Ascorbate has been implicated as the preferred substrate in the export of electrons (2). Ascorbate regeneration from dehydroascorbate is strongly dependent on glycolysis in red blood cells (RBCs) and occurs via intracellular reduced glutathione (GSH) in a process involving both NADH and NADPH (3). Moreover, a definite interrelationship of the various sulfhydryl populations on the RBC membrane in the plasma and on the plasma proteins (albumin) has been suggested to be operating so that the cellular redox balance affects the extracellular environment (4).

Diabetes causes both enhanced oxidative stress and impaired regeneration of protective antioxidants (5). Abnormalities in ascorbic acid metabolism (6), impaired GSH synthesis and thiol transport (7), and significant defects of chain-breaking antioxidant blood protection (8) have all been described in patients with both type 1 and type 2 diabetes. Increased cytosolic reductive stress associated with an increased ratio of NADH to NAD+ is a candidate mechanism of early diabetes-induced glomerular dysfunction, manifested as increases in blood flow, glomerular filtration rate, and microalbuminuria (9).

In the present study, we have measured the erythrocyte-mediated ferricyanide reduction system with intact erythrocytes from type 1 diabetic patients with or without complications from microangiopathy, from their non-diabetic siblings, and from healthy control subjects. We wished to explore the status of transplasma membrane redox activity in the patients with diabetes, its relationship with diabetic complications, and the contribution of familial factors to the electron transfer rate.

RESEARCH DESIGN AND METHODS

Patients with type 1 diabetes (n = 30) and control age-matched nondiabetic subjects (n = 30) were studied (Table 1). Diabetic patients had been treated with at least 2 daily insulin injections from the time of diagnosis (disease duration 20 ± 8 years). None received medical treatment except insulin (0.65 ± 0.13 U/kg) or possibly antihypertensive drugs. The group consisted of 10 patients without diabetic complications, 10 patients with retinopathy (background or proliferative, determined by fluorescein angiography), and 10 patients with nephropathy. Among the patients with nephropathy, 1 was on dialysis (serum creatinine levels >133 µmol/l), 4 had persis-
tent macroalbuminuria (urinary albumin excretion rate [UAER] >200 µg/min and serum creatinine <133 µmol/l), and 5 had persistent microalbuminuria (defined as a UAER >20 µg/min in 2 of 3 consecutive 24-h urine collections within 6 months in the absence of a urinary tract infection or heart failure).

Two of the patients with retinopathy and 6 of the patients with nephropathy were taking drugs, including ACE inhibitors, calcium antagonists, and/or vasodilators.

We compared 36 nondiabetic normotensive siblings of the diabetic patients (age 39 ± 13 years, BMI 25 ± 4 kg/m², and mean blood pressure 92 ± 14 mmHg) with 36 matched control subjects (39 ± 10, 25 ± 4, and 93 ± 10). Twelve were siblings of probands with nephropathy and 16 of probands with retinopathy and normalalbuminuria. No study subject was taking antioxidant vitamins at the time of blood sampling. All subjects gave their informed consent to participate in the study.

Participants were interviewed with regard to their own and familial medical history. They underwent a routine clinical assessment. Height and weight were measured (in indoor clothing without shoes). The mean blood pressure was calculated as the diastolic pressure plus one-third of the pulse pressure.

Biochemical analyses

After an overnight fast, blood was collected in heparin-rinsed syringes and immediately centrifuged at 54g for 9 min at 20°C to remove platelet-rich plasma. Packed RBCs were washed 3 times in 10 volumes of phosphate-buffered saline, which consisted of 120 mmol/l NaCl, 5 mmol/l KCl, 1 mmol/l MgSO₄, 16 mmol/l NaH₂PO₄, and 5 mmol/l glucose (pH 7.4), and centrifuged at 1,320g for 5 min at 4°C.

To measure ferricyanide reduction, 0.5 ml of the packed cells were diluted to 5 ml with phosphate-buffered saline containing 1 mmol/l ferricyanide and incubated under magnetic stirring in a water bath at 37°C. At 5, 10, 20, and 40 min, 0.3-ml aliquots of the cell suspension were removed and centrifuged at 3,110g for 4 min at 4°C (10). An aliquot of 0.05 ml of the supernatant was measured by the assay of Avron and Shavit (11). Ferrocyanide was determined from its absorbance at 535 nm with 4,7-diphenyl-1,10-phenanthroline and sulfonated as the color-developing agent against a 0 time blank. The initial rate of ferrocyanide generation was expressed relative to the real packed cell volume (determined by an automated cell counter) as micromoles per milliliter packed RBCs per h. The within- and between-run coefficients of variation were 6 and 9%, respectively.

The level of erythrocyte GSH was measured by the method of Buteler et al. (12) and expressed as milligrams per milliliter RBCs. Plasma sulfhydryl (SH) groups were measured using fresh plasma and the colorimetric assay described by Ellman (13). The cytosolic ratio of NADH to NAD⁺ was inferred from the ratio of plasma lactate to pyruvate. Plasma glucose, fructosamine, lactate, and pyruvate were measured by standard enzymatic methods (reagents from Boehringer Mannheim, Mannheim, Germany). HbA₁c was evaluated by the Bio-Rad Diamat Fully Automated Glycosylated Hemoglobin Analyzer System (Bio-Rad). Albunin was quantified by the kinetic immunonephelometric method with an automated instrument (Behring Institute nephelometer and reagents; Scoppito, L'Aquila, Italy).

Statistical analysis

All data are expressed as means ± SD, except plasma lactate and pyruvate, which are given as median and range. Results were analyzed by a commercial software package.
Ferricyanide reduction in diabetic nephropathy

Figure 1— Individual distribution of erythrocyte rate of ferricyanide reduction in diabetic subgroups and control subjects. Bars identify mean values.

(StatView, Abacus Concepts, Berkeley, CA) using a Students unpaired t test or for not normally distributed data, a Mann-Whitney U test. A χ² test was used to compare prevalence among groups. A P value <0.05 was considered significant. Correlations were sought by using stepwise multiple linear regression analysis. Before regression analysis, nonparametric values were log-transformed. Retrospectively, the patient sample size had a 65% power to identify differences (P < 0.05) in velocity of ferricyanide reduction of a 30% magnitude (14).

RESULTS — Type 1 diabetic patients and control subjects were well matched for age, sex distribution, BMI, and arterial blood pressure (Table 1). The diabetic patients had significantly elevated levels of fasting plasma glucose, fructosamine, and glycated hemoglobin (HbA₁c) compared with the control subjects. In type 1 diabetic patients, both erythrocyte GSH levels and plasma SH groups were significantly lower than those in control subjects (Table 1). Changes in erythrocyte-mediated ferricyanide reduction were not observed in the diabetic group compared with the control group. The patients with a known history of diabetic nephropathy (n = 10) were compared with 10 type 1 diabetic patients of similar age, sex distribution, BMI, and long duration of diabetes, who had retinopathy but normal UAERs (Table 2). The 2 groups were comparable with regard to plasma glucose, fructosamine, HbA₁c, lactate-to-pyruvate ratio, and plasma thiols. In the patients, the only significant differences were the lower erythrocyte concentrations of GSH and the higher ferricyanide-reducing capacity in the nephropathy group compared with the retinopathy group (Table 2). Individual distribution of erythrocyte ferricyanide reduction capacity in diabetic subgroups and control subjects is shown in Fig. 1.

No significant difference emerged when comparing the rate of ferricyanide reduction of patients with complications who were taking drugs (n = 8, 13 ± 6 µmol · ml⁻¹ · h⁻¹) with those who were not (n = 12, 15 ± 5 µmol · ml⁻¹ · h⁻¹).

The remaining 10 patients without diabetic complications were younger than the groups with complications and showed both shorter duration of diabetes (14 ± 9 years, P < 0.05) and lower glycated hemoglobin levels (P < 0.05). These 10 patients were compared with 10 matched healthy control subjects (Table 3). They differed from the control group in glycemic control and erythrocyte GSH. No difference was detected when comparing siblings of diabetic patients with healthy control subjects (ferrocyanide 13 ± 6 vs. 14 ± 5 µmol · ml⁻¹ · h⁻¹, respectively) as well as siblings of patients with nephropathy with siblings of patients with retinopathy and normal albuminuria (ferrocyanide 14 ± 5 vs. 13 ± 5 µmol · ml⁻¹ · h⁻¹, respectively; other data not shown). Erythrocyte GSH showed significant negative correlation with fasting plasma glucose (r = 0.3, P = 0.05) and fructosamine (r = 0.3, P = 0.01). In the diabetic group without complications, a significant relation was observed between ferricyanide reduction capacity of erythrocytes and plasma lactate concentration (r = 0.8, P = 0.01; Fig. 2), whereas no relation was present in the diabetic groups with microangiopathic complications. Among all patients with type 1 diabetes, multiple

Table 3— Clinical and metabolic characteristics of 10 type 1 diabetic patients without complications versus 10 matched healthy volunteers

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Type 1 diabetic subjects without complications</th>
<th>Control subjects</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex (M/F)</td>
<td>4/6</td>
<td>3/7</td>
<td>—</td>
</tr>
<tr>
<td>Age (years)</td>
<td>29 ± 10</td>
<td>30 ± 8</td>
<td>—</td>
</tr>
<tr>
<td>Duration of diabetes (years)</td>
<td>14 ± 9</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Insulin dose (U/kg)</td>
<td>0.6 ± 0.1</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>24 ± 2</td>
<td>22 ± 2</td>
<td>—</td>
</tr>
<tr>
<td>Mean blood pressure (mmHg)</td>
<td>87 ± 10</td>
<td>85 ± 11</td>
<td>—</td>
</tr>
<tr>
<td>Plasma glucose (mmol/l)</td>
<td>13 ± 4</td>
<td>5 ± 0.4</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Fructosamine (µmol/l)</td>
<td>348 ± 47</td>
<td>229 ± 11</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>HbA₁c (%)</td>
<td>7.4 ± 1.3</td>
<td>5.2 ± 0.4</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Plasma lactate (mmol/l)</td>
<td>1.5 (1.14–2.3)</td>
<td>1.7 (1.3–2.8)</td>
<td>—</td>
</tr>
<tr>
<td>Plasma pyruvate (mmol/l)</td>
<td>0.05 (0.01–0.1)</td>
<td>0.04 (0.01–0.1)</td>
<td>—</td>
</tr>
<tr>
<td>GSH (mg/ml RBC)</td>
<td>0.70 ± 0.11</td>
<td>0.88 ± 0.16</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>SH groups (µmol/l)</td>
<td>447 ± 51</td>
<td>438 ± 53</td>
<td>—</td>
</tr>
<tr>
<td>Plasma albumin (mg/dl)</td>
<td>4,626 ± 323</td>
<td>4,782 ± 404</td>
<td>—</td>
</tr>
<tr>
<td>Ferricyanide (µmol · ml⁻¹ · h⁻¹)</td>
<td>13 ± 4</td>
<td>13 ± 5</td>
<td>—</td>
</tr>
</tbody>
</table>

Data are n, means ± SD, or medians (range).
logistic regression of the electron transport capacity gave positive correlations with urinary albumin excretion and plasma creatinine and a negative correlation with plasma thiol concentration (multiple $r = 0.6$, $P < 0.01$; Table 4). Plasma SH groups correlated negatively with disease duration ($r = 0.4$, $P = 0.01$) and circulating albumin ($r = 0.4$, $P = 0.01$). Plasma albumin concentration decreased with increasing duration of diabetes ($r = 0.4$, $P < 0.05$).

CONCLUSIONS — The major novel observation in our study of type 1 diabetic patients with or without microangiopathic complications, their siblings, and healthy control subjects is the finding that the rate of ferrocyanide generation by erythrocytes is significantly elevated in diabetic patients with nephropathy. The abnormality is peculiar to the nephropathy group and seemingly not contributed by familial or hereditary components because the electron flow was normal in siblings. The activity of the erythrocyte transmembrane-reducing system has been measured in a clinical setting now for the first time. Previous studies examined the mechanism by which human erythrocytes transfer electrons to extracellular ferricyanide and the ultimate source of the reducing equivalents (1–3,10,15). The addition of an oxidating agent such as ferricyanide or Ellman's reagent to the medium of erythrocyte suspensions has been suspected of inducing the thiol-disulfide exchange reaction that occurs on the esofacial surface of the membrane (4). As a result of the oxidative stimulus at the membrane surface, the cytosolic GSH pool is both oxidized and depleted to regenerate the disulfide formed. This reaction requires electrons and energy from the cell. Few compounds have been found to act on transplasma membrane ferricyanide reduction by intact human erythrocytes (15): dehydroascorbate supplementation, inhibitors of glycolysis, p-chloromercuriphenyl sulfonate (suggesting the involvement of SH groups on the outside of the membrane in the electron transfer process).

In diabetes (as in other diseases involving oxidative stress) the plasma and membrane may be observed to have lower thiol levels than in normal healthy volunteers.

Indeed, our type 1 diabetic patients had lower levels of both erythrocyte GSH and plasma sulfhydryl groups. Reduced GSH concentration in erythrocytes showed negative correlations with short- and mean-term metabolic control parameters, such as fasting plasma glucose and fructosamine, rather than with long-term HbA1c. Discrepancies among reports with regard to erythrocyte GSH in diabetes and its relationship with metabolic parameters and diabetic complications (16–19) usually have been ascribed to differences in glycemic control, which was on average worse in our complicated groups. However, this finding does not explain the difference in erythrocyte GSH content between the nephropathy and retinopathy groups who showed similar metabolic control. With regard to the low GSH levels of the patients without complications, abnormalities of chain-breaking antioxidant status and erythrocyte GSH depletion have been observed in patients with either type 1 and type 2 diabetes, both at disease onset and in later stages (8,16). No relationship has been found between the level of GSH in erythrocytes and the duration of diabetes (17). Only a follow-up will tell us whether the patients with low levels of GSH in early stages of the disease are more prone to diabetic nephropathy.

The ratio of plasma lactate to pyruvate, which is a reliable parameter of the cytosolic ratio of free NADH to NAD$^+$ (20), has been considered a marker of hyperglycemic pseudohypoxia (21).

In our patients without diabetic complications, cytosolic NADH was the major determinant of erythrocyte electron transfer capacity (Fig. 2). In contrast, in patients...
with diabetic complications, the close relationship between NADH generation and transmembrane electron passage seemed to be lost. Whereas patients with retinopathy alone still had normal activity of the erythrocyte-reducing system, patients with nephropathy showed significantly increased activity related only to renal function parameters (plasma creatinine and urinary albumin) and plasma thiols.

The possibility that chronic renal failure and/or dialysis treatment could have contributed to our findings was excluded by the composition of the nephropathy group: no patients had renal failure but one. To exclude any measurable contribution of the antihypertensive treatment to the increased rate of ferricyanide reduction in diabetic nephropathy, we compared the patients with complications who were taking drugs and those who were not: no significant difference emerged. On the other hand, the indicated drugs have been reported to have no adverse effect on glucose metabolism (22); moreover, only one patient was receiving the SH-containing captopril, which seemed to preserve myocardium Q10 and cytochrome oxidase activity (23).

In living organisms, thiols, thiocyanates, and disulfides are interdependent and constitute elements of an electron transfer system along with metalloproteins (24). Parameters controlling the rates of electron transfer reactions have been investigated by using a range of model systems (25). Earlier work established the rate dependence on driving force and distance. In addition, the medium separating the donor and acceptor also has to play an important role. The reorganization energy (λ) may be another important factor for many systems, owing to reorientation of water molecules. In the absence of diabetic complications, the rate of electron transfer was, as expected, proportional to the driving force (NADH-to-NAD ratio). At both equal oxidative stimulus and driving force, changes in the transfer matrix element have to account for the loss of the proportionality observed in the diabetic groups with microangiopathy.

The electron flow among the redox centers is in part controlled by protein components (covalent switching). The development of diabetic complications may be significant for electron transfer rate, owing to irreversible chemical modifications and cross-links in proteins (26) or reorientation of solvent water molecules (to determine if the reorientation of solvent water molecules is the accelerating factor in diabetic nephropathy). Which dynamics of the intervening medium really accelerated the electron transfer along functional pathways is still to be examined.

We observed that if too many electrons are passed on at a time, harmful free radicals result that are candidate mechanisms involved in the process of renal damage. The data presented support the hypothesis that the transmembrane-reducing system of the erythrocytes is selectively involved in diabetic nephropathy, where erythrocyte GSH is also depleted. Future studies are needed to investigate the underlying mechanisms and the chronological order in which these abnormalities develop. Erythrocyte transmembrane electron transfer may be a useful tool to directly monitor cellular redox state and, moreover, to evaluate efficacy of preventive interventions.

Acknowledgments — We acknowledge the superb technical assistance of Vincenzo Cinapi and Stefano Quilici.

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