Relationship between erythrocyte GLUT1 function and membrane glycation in type 2 diabetes

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ABSTRACT

This paper investigates the effect of glycation on glucose transport in erythrocytes. Glucose transporter function, numbers and erythrocyte phosphorylation rates are simultaneously studied using 30 Caucasian patients with diabetes and 30 Caucasian control volunteers (mean±SD where $P \leq 0.05$; age 48±8 *vs.* 45±8 years [ns]; body mass index [BMI] 31±7 *vs.* 27±5 [$P=0.035$]; blood glucose 12±7 *vs.* 5±0.6 mmol/L [$P=0.001$]; HbA1c 8±2 *vs.* 5±0.3% [$P=0.0001$]; fructosamine 336±64 *vs.* 237±16 µmol/L [$P=0.0001$]; disease duration 13±11 years, respectively). Significant differences were found for glucose transporter function, with 3-O-methylglucose uptake rates (108±49 *vs.* 146±55 µmol/L/sec/10^{12} cells [$P=0.010$]); D-glucose influx (64±30 *vs.* 117±45 µmol/L/sec/10^{12} cells [$P=0.0001$]); and D-glucose net transport (31±22 *vs.* 74±55 µmol/L/sec/10^{12} cells [$P = 0.0001$]). No differences were found for phosphorylation rates using 2-deoxyglucose (33±17 *vs.* 38±12 µmol/L/sec/10^{12} cells [$P=0.194$]). The number of functional transporters using cytochalasin B studies measured via B_{max}, was not found to be significantly different between the groups (195±139 *vs.* 264±174 [$P=0.206$]). However, K_d was lower for those with diabetes, suggesting higher binding affinity (12±11 *vs.* 32±25 nmol/L [$P=0.006$]). A negative correlation between HbA1c and D-glucose influx involving both groups was found ($r=-0.670$, $P=0.0001$). Glucose transport is shown to be decreased in people who have diabetes compared to normoglycaemic volunteers, whereas the number of glucose transporters is apparently unchanged; however, affinity for binding is increased.

KEY WORDS: Diabetes.
Membrane glycation.
GLUT1 function.

Introduction

The glucose transporter proteins play a pivotal role in glucose metabolism and they are responsible for basal glucose uptake. The pattern of expression of the GLUT transporters is related to their different roles in glucose metabolism and varies in different tissues.[1]

It is generally accepted that poor glycaemic control contributes to the development of diabetic complications, as reported by the DCCT and UKPDS studies.[2,3] Glycation can alter protein morphology and can affect physiological function.[4] GLUT1 has already been found to be susceptible to glycation both *in vitro* and *in vivo*[5] but this has not been fully examined in relation to function or in volunteers with diabetes.

Studies indicate differences in erythrocyte glucose uptake; however, there are inconsistencies in the published data, which may be in part due to the small numbers in some studies.[6,7] Glycation provides a potential molecular mechanism by which GLUT1 function may be altered in diabetes mellitus, which may contribute to tissue damage, as it is found in non-insulin-dependent tissues. Pathogenic factors (e.g., protein glycation, tissue oxidation and endothelial function) in diabetes are interrelated.[8]

The study aims to show that glycation has a role to play in reducing transporter glucose uptake which complicates the ability to achieve good glycaemic control in type 2 diabetes.

Materials and methods

Study subjects

Thirty Caucasian type 2 diabetic patients were recruited during routine visits to the clinic at the Wolverhampton Diabetes Centre. Thirty Caucasian normoglycaemic volunteers were also recruited.

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Inclusion criteria

The patient group criteria were as follows: generally good glucose control (HbA1c 5–12%, random blood glucose 6–15 mmol/L, fructosamine 245–460 µmol/L), have type 2 diabetes, and be registered at the Diabetes Centre at the Royal New Cross Hospital, Wolverhampton. Controls were non-diabetic, with no diseases of the heart, liver or kidneys (HbA1c 4–5%, random blood glucose 3–5 mmol/L, fructosamine 200–257 µmol/L).

Exclusion criteria

Patients with diabetes plus chronic kidney or liver disease, or who had suffered a myocardial infarction or stroke were excluded from the study.

The Royal New Cross Hospital Medical Ethics Committee

approved the study. After informed written consent, non-fasting 20 mL blood samples were drawn into sodium heparinised tubes (10 iu/mL).

Preparation of red blood cells
Blood samples were centrifuged at 3000 rpm for 10 min. Plasma was collected into a sterile universal and the buffy coat was discarded. The cells were washed (x3) with 10 mL phosphate-buffered saline (PBS; pH 7.4). After the final wash, the supernatant was discarded and the red cells were resuspended to a haematocrit value of 20%. After washing, a cell count was performed to observe white cell depletion and red cell count for subsequent calculations.

Preparation of red cell membranes
Washed red cells in PBS were added to lysis buffer with phenyl-methyl-sulphonyl fluoride (PMSF; 10 mg/mL in ethanol) and left on ice for 20 min. The cells were then ultracentrifuged at 20,000 rpm at 6°C for 20 min. The procedure was repeated until a white pellet was obtained, and this was stored at –70°C.

Measurement of glucose efflux, influx and net movement in RBCs
The principle of the assay was to measure glucose influx in glucose-depleted, washed erythrocytes, and glucose efflux in glucose-loaded erythrocytes resuspended to a haematocrit value of 20% using either PBS (pH 7.4) or 10 mmol/L glucose/PBS (pH 7.4). Net movement was measured where the wash and incubation medium was 10 mmol/L glucose/PBS (pH 7.4) solution. The cells were incubated in a water bath for 30 min at 37°C with periodic shaking. After incubation, the sample was centrifuged at 13,000 rpm for 2 min and the supernatant retained to measure glucose (Technicon RA 1000 meter) (in triplicate). The glucose uptake was calculated from the difference between the starting glucose concentration of the incubation buffer and the final supernatant glucose concentration after incubation. Within assay and between assay coefficients of variation (CV) were measured as follows: glucose influx (n=20, 0.74%; n=10, 0.98%, respectively), glucose efflux (n=10, 1.03%; n=20, 1.15%, respectively) and net movement (n=20, 1.09%; n=10, 1.16%, respectively).

Measurement of 3-O-methyl-[3H]-glucose uptake
This was performed using the method of Devivo *et al*.[10] The within and between assay CV of 3-O-methylglucose expressed as µmol/L/10^{-12} cells were measured as follows: time 0 (n= 36, 2.65%; n=10, 3.77%, respectively), 2 sec (n=10, 4.09%; n=20, 8.74%, respectively), and 5 sec (n= 36, 1.76%; n=10, 1.69%, respectively).

Measurement of 2-deoxy-D-[1-3H]-glucose phosphorylation
This was performed using the method of Kletzien *et al*.[11] The within and between assay CVs of 2-deoxyglucose phosphorylation rate were expressed as µmol/min/10^{12} cell (n=33, 3.76%; n=10, 2.43%, respectively).

Measurement of glucose transporter numbers – cytochalasin B assay
This was performed using the method of Comi *et al*.[12] Scatchard plot analysis was used to determine B_{max} (transporter numbers) and K_d (dissociation constant, as a measure of GLUT1 binding affinity). The within and between assay CVs were measured at 50 pmol/mg (n=30, 2.86%; n=10, 4.51%, respectively), 200 pmol/mg (n=30, 3.12%; n=10, 3.09%, respectively), 450 pmol/mg (n=30, 2.12%; n=10, 2.74%, respectively) and at 900 pmol/mg (n=30, 1.97%; n=10, 2.47%, respectively).

Erythrocyte membrane preparation
This was performed using the method of Bilan *et al*.[5] Erythrocytes (4 mL) were added to 30 mL lysis buffer and left on ice for 15 min. This was centrifuged at 20,000 rpm at 4°C for 20 min (plus a deceleration time of 4 min). Four centrifugal washes produced a white or light pink pellet which was stored at –70°C.

Protein determination
Protein was determined using the Lowry method.[13] Within and between assay CVs of the enhanced Lowry procedure were measured (n=10, 0.01%; n=30, 0.04%, respectively).

Membrane preparation for total glycation measurement
Cell membranes were solubilised in sample preparation buffer (SPB) and left on ice for 20–30 min. The samples were centrifuged for 10 min at 12,500 rpm at 4°C and a supernatant dilution (1 in 4) in 1% sodium dodecyl sulphate (SDS) was prepared for protein estimation.

Total membrane glycation measurement
The method was modified from the methods of Johnson *et al*.[14] and Kricka *et al*.[15] using periodate to determine total erythrocyte membrane glycation. A test reagent (0.2 mol/L Na_2CO_3, 0.5 mmol/L nitro blue tetrazolium [NBT]; pH 10.35) and blank reagent (0.2 mol/L Na_2CO_3, 0.5 mmol/L NBT, 62.5 mmol/L sodium tetraborate) were prepared. To solubilised the membrane, the test reagent was added and incubated at 37°C for 20 min, and then the absorbance was read at 530 nm. The procedure was repeated with the blank reagent. The difference between the test and the blank readings was proportional to the level of glycation. Protein concentration was expressed in mg. A fructosamine calibrator (Sigma Aldrich) was used to prepare a standard curve. This was sufficient as long as the batch test and blank reagents prepared were used for the assay. The within assay CV range was 4.7–5.3% and the between assay CV range was 8.8–10.3%.

Data analysis
Results were expressed as mean±standard deviation (SD). Statistical analysis was carried out using SPSS for Windows, version 10.0 (SPSS, Chicago, Illinois, USA). Analysis of variance (ANOVA) correlation and two-tailed bi-variant coefficients were determined. The relationship of independent variables on any dependent variable was examined using linear and multivariate regression analysis. Statistical tests were considered significant at $P \leq 0.05$.

Results

Clinical characteristics
Body mass index, HbA1c, fructosamine and blood glucose were found to be significantly higher in Caucasian patients who had diabetes compared to the control group (Table 1).

Table 1. Clinical characteristics of the patient and control groups.

Category	Patients (n=30, mean±SD)	Controls (n=30, mean±SD)	P value
Disease duration (years)	13±11		
Age (years)	48±8	45±8	0.992
BMI (k/gm^2)	31±7	27±5	0.038
Blood glucose (mmol/L)	12±7	5±0.6	0.001
HbA1c (%)	8±2	4.9±0.9	0.0001
Fructosamine (µmol/L)	324±63	240±29	0.0001
Total membrane glycation (µg/mg)	8±2	6±3	0.01

Table 2. Effect of diabetes on glucose transporter function.

Variable	Patients (n=30, mean±SD)	Controls (n=30, mean±SD)	P value
D-glucose efflux (µmol/L/min/10^{-12} cells)	54±21	57±38	0.709
D-glucose influx (µmol/L/min/10^{-12} cells)	64±30	117±45	0.0001
D-glucose net movement (µmol/L/min/10^{-12} cells)	31±22	74±55	0.0001
2-deoxyglucose phosphorylation rate (µmol/L/min/10^{-12} cells)	33±17	38±12	0.194
2-DGRATIO (%)	56±18	58±9	0.509
3-O-methylglucose uptake (2 sec; µmol/L/min/10^{-12} cells)	10±49	146±55	0.01
3-O-methylglucose uptake (5 sec; µmol/L/min/10^{-12} cells)	228±104	277±107	0.104

2-DGRATIO: 2-deoxyglucose phosphorylation ratio between uptake and phosphorylation rate

Table 3. The effect of diabetes on cytochalasin B binding activity.

Variable	Patients (n=30, mean±SD)	Controls (n=30, mean±SD)	P value
CytB_{max} (pmol/mg)	195±139	264±174	0.206
CytK_d (nmol/L)	12±11	32±25	0.006
Total membrane glycation (µg/mg)	8±2	6±3	0.01

Age was not significantly different. The groups were matched as close as possible for gender (control group: 14 females, 16 males; patient group: 11 females, 19 males).

Effect of diabetes on glucose transporter function

Glucose transporter function was assessed using D-glucose influx, efflux and net uptake, as well as 3-O-methylglucose uptake and 2-deoxyglucose phosphorylation rates and ratio. The results comparing the patient and control groups can be seen in Table 2. Patients had significantly lower rates when compared to controls (P<0.05) for D-glucose influx, net transport rates and 3-O-methylglucose transport rate (3-OMG) at 2 sec. Figure 1 shows the relationship between D-glucose influx, efflux and net erythrocyte transport in the patient and control groups.

D-glucose influx showed a negative correlation with HbA1c when the groups were combined (r=–0.67, P=0.0001; Fig. 2). No correlation was seen with total membrane glycation (r=–0.15 P=0.370).

A significant strong positive correlation was demonstrated between erythrocyte glucose net transport and influx rates within the control group, which was expected as erythrocyte influx generally moved towards equilibrium in a zero-trans measurement *in vitro* (r=0.68, P=0.0001; Fig. 3.).

A significant positive correlation between 3-OMG at 5 sec and 2-deoxyphosphorylation rates in the control group only was observed (r=0.43, P=0.017).

A strong positive correlation existed between 2-deoxyphosphorylation ratio and efflux (r=0.624, P= 0.001) in the patient group only.

Effect of diabetes on cytochalasin B binding activity

Cytochalasin B binding was used to determine whether or not glycation could affect glucose transporter function as it is a highly specific competitor inhibitor and ligand of the glucose binding domain. Membranes isolated from both groups were incubated without and with D-glucose, using Scatchard analysis to determine maximum binding (B_{max}) and the dissociation constant (K_d) or the affinity of binding between cytochalasin B with the glucose transporter complex. In the presence of glucose, this curve represented non-specific binding as glucose readily displaced cytochalasin B at the glucose transporter site. B_{max} was not significantly greater in the control group. K_d was found to be

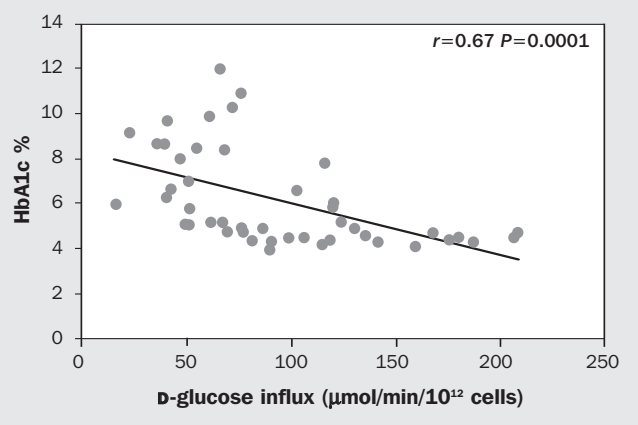

Fig. 1. Correlation between HbA1c and erythrocyte D-glucose influx rate (n=60).

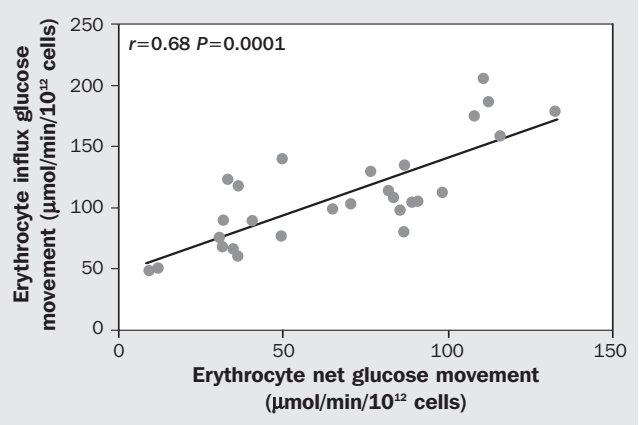

Fig. 2. Correlation between erythrocyte D-glucose net and influx movement.

significantly (P<0.01) lower in the patient group (Table 3), implying higher binding affinity.

Relationship between HbA1c and total erythrocyte glycation

Total membrane glycation was greater, as expected, in the patients when compared to the controls (Table 1). In addition, the method correlated well with HbA1c (patient and control groups: r=0.506, P=0.0001; patients only: r=0.728, P=0.001). This suggests that the erythrocyte membrane transporter is glycated at a similar rate to haemoglobin.

Discussion

The aim of this study was to investigate the pathophysiological role of hyperglycaemia in the development of diabetic complications. This involved measuring its effect on erythrocyte membrane glycation and glucose transporter function and numbers. Markers of erythrocyte glucose transport function included D-glucose influx, efflux, net transport rates, 3-O-methylglucose and 2-deoxyglucose phosphorylation rates. Cytochalasin B binding was used to determine transporter numbers (B_{max}) and binding affinity (via K_d). HbA1c, fructosamine and total membrane glycation were measured in 30 patients with diabetes and 30 control Caucasian volunteers to assess the relationship with hyperglycaemia. A cross-sectional outlook was used for correlation analysis in order to look at the interrelationship between the different markers.

Glycation and glucose transport

Glucose is an essential metabolic substrate of all mammalian cells and the human erythrocyte membrane contains the GLUT1 transporter in abundance.[16] This investigation into erythrocyte glucose transport using several methods has demonstrated significant differences in glucose transport rates between patients who have diabetes and control volunteers. Measuring D-glucose influx and 3-O-methylglucose uptake at 2 sec showed differences between the two groups; influx was significantly reduced in the diabetic patients. This finding agrees with Nielsen *et al.*,[17] who measured erythrocyte tritiated glucose uptake in the presence of low insulin infusion in type 2 diabetic patients and in control volunteers. They concluded that an abnormality in uptake occurred in the presence of basal and high insulin concentrations, and it was evident in diabetes that the ability of glucose to stimulate its own uptake was impaired.

The D-glucose influx methodology was designed to mimic mammalian physiological conditions and showed that net transport was reduced in people who have diabetes, which supports the impaired influx observations as net transport could not be achieved successfully without sufficient influx. This simple assay provided further evidence of abnormal glucose transporter function. This was in the absence of impaired metabolism as measured by 2DG phosphorylation rates.

HbA1c correlates with intracellular glucose concentration and may be a more precise marker when compared to the total membrane glycation method in assessing the effect of hyperglycaemia, due to the higher precision of the assay. This may explain its negative correlation (r value) with glucose influx and no significant correlation with total membrane glycation. These results suggest that decreased erythrocyte glucose transport may be related to increased glycation. Glycation could also explain the observations of Hu *et al.*,[18] who demonstrated a structural change in the glucose transporter GLUT1 outer domain in Asian Chinese patients who had type 2 diabetes. However, further investigation is necessary in view of the identification of type 2 diabetes susceptibility genes[19] in the Indo-Asian population.

Comparison of glucose uptake

Significantly lower 3-OMG uptake was observed in the patient group at the 2 sec interval but not at the 5 sec incubation period. Comparing the 3-OMG and cold D-glucose methodologies, greater differences were seen with the cold glucose, which may relate to the difference in structure and binding affinity for the two compounds. Studies on the binding affinities of D-glucose and 3-O-methylglucose demonstrate that experimental conditions have an impact on the outcome and could be a possible explanation to the controversy surrounding erythrocyte glucose uptake. Kawano[20] observed that high concentrations of 3-O-methylglucose can encourage a concentration-dependent increase in [3H]-3-O-methylglucose transport into cells, which could explain the work of Bistritzer[6] who observed an increase in erythrocyte 2 sec

3-O-methylglucose uptake in nine diabetic patients compared with seven healthy individuals.

Cytochalasin B binding

Cytochalasin B has been used to identify glucose transporter binding sites.[21–23] The number of functional glucose transporters was not found to be significantly different between people with and without diabetes. However, binding affinity was significantly higher in the patient group. This is in contrast to the findings of Bilan's *in vitro* studies[5] in which glycated membranes showed lower B_{max} and no significant relationship between binding affinity and glycation. One possible explanation could be the lack of variability of glycation, as all patients had good glycaemic control, whereas the *in vitro* glycation employed by Bilan is likely to have produced more variability.

The *in vitro* studies performed by Bilan,[5] where membranes were glycated over a number of days with 200 mmol/L D-glucose compared to controls (without D-glucose incubation), gave higher B_{max} and K_d values but in the same order when compared to the present *in vivo* study. Bilan also found that glycation altered B_{max} rather than K_d. Both studies imply that glycation affects glucose binding to GLUT1.

The present study demonstrates that diabetics and non-diabetics have similar erythrocyte glucose metabolism in the form of phosphorylation rates, D-glucose efflux rates and a similar number of transporters. However, diabetics show abnormal glucose uptake and an inverse relationship with erythrocyte membrane glycation, which suggests that transporter glycation may be a possible cause. This work supports Hu *et al.*[18] who also observed a decrease in glucose entry in the erythrocyte membranes in Chinese type 2 diabetic patients. This was explained by a change in structure of GLUT1 (mainly the outer domain of the glucose transporter). In light of the recent study by Peyroux *et al*.[24] of advanced glycation end-product (AGE) inhibitors in animals with diabetes, it is suggested that more work is required to develop therapeutic interventions involving glycation prevention to safeguard against diabetic complications, in order to improve the long-term quality of life for type 2 diabetics. Ultimately, this would also reduce overall healthcare costs. □

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