Kinetic Behavior of the Erythrocyte Sodium-Lithium Countertransporter in Nonnephropathic Diabetic Twins

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Elevated erythrocyte sodium-lithium countertransport activity occurs in diabetes and may be genetically mediated. The relation of this abnormality to the disease and its complications is unclear. To remove confounding genetic factors and the impact of complications, we studied sodium-lithium countertransport activity together with its kinetic components, maximal rate of turnover ($\dot{V}_{max}$) and external affinity for sodium (k_{Na}), in identical-twin pairs discordant for insulin-dependent diabetes who were normotensive and had no evidence of nephropathy. Fifteen twin pairs were studied along with the same number of healthy control subjects matched with the twins for gender, age, and body mass index. Clinical and laboratory characteristics of the twins and controls were similar, with the exception that whole blood glucose and glycated hemoglobin concentrations were higher in diabetic twins ($P < .001$). Comparison of countertransport activity between nondiabetic and diabetic twin groups failed to uncover any significant differences ($P = .30$, Wilcoxon). Similarly, there were no differences in countertransport activity between the nondiabetic twin group and the controls ($P = .38$, Mann-Whitney). Furthermore, no associations were noted between residual activity values and residual data of any of the other clinical or laboratory characteristics measured. Comparison of $\dot{V}_{max}$ between nondiabetic and diabetic twin groups showed a significant elevation in the diabetic twins (0.515 + 0.220 *v* 0.439 + 0.229 mmol Li/L RBC · h, $P = .049$, paired *t* test). By contrast, no significant differences were noted between the nondiabetic twin group and the controls ($P = .15$, unpaired *t* test). Comparison of k_{Na} between nondiabetic and diabetic twin groups found no significant differences in k_{Na} ($P = .42$, Wilcoxon). Similarly, there were no differences in k_{Na} between nondiabetic twins and controls ($P = .14$, Mann-Whitney). Neither the residual data for $\dot{V}_{max}$ nor k_{Na} showed any association with the residual data of any of the other clinical or laboratory characteristics measured. When intertwin correlations were examined, all three parameters describing the behavior of the sodium-lithium countertransporter showed significant intertwin correlations (activity, $r = .51$, $P = .04$; $\dot{V}_{max}$, $r = .82$, $P = .001$; k_{Na}, $r = .76$, $P = .001$). In conclusion, the diabetic state has a small effect on the $\dot{V}_{max}$ of the sodium-lithium countertransporter. Failure to consider the complex nature of the activity measurement is likely to have been partly responsible for earlier confusion with regard to the effect of diabetes on the countertransporter, since experimental conditions varied between studies and individual kinetic components were not measured. The associations between twins in this study with respect to $\dot{V}_{max}$ and k_{Na} indicate a genetic influence on both constants of the countertransporter. $\dot{V}_{max}$ appears also to be sensitive to certain as yet unidentified environmental factors.

ABNORMAL BEHAVIOR of the erythrocyte membrane sodium-lithium countertransporter is associated with diabetes.[1,2] However, problems have arisen in the interpretation of this abnormality, which could be linked either to the disease itself and/or to its complications.[3] The failure of many studies to consider or separate the confounding influences of genetics and disease processes has caused confusion.[3] Part of the reason for this may be the complex mix of genetic and environmental influences on the countertransporter.[4,5] Another reason may lie within the measurement of sodium-lithium countertransport activity itself. Until recently, the activity value was thought to represent the maximal turnover rate ($\dot{V}_{max}$) of the countertransporter. However, there is a basic weakness in the reliance on countertransport activity as anything other than a rate of lithium efflux determined under specified conditions, since there is good evidence that countertransport activity is a composite expression of the $\dot{V}_{max}$ and the external affinity for sodium (k_{Na}).[6]

The present study was performed to determine whether the diabetic state has an effect on the sodium-lithium countertransporter that is distinct from influences of an environmental or genetic nature? To control for environmental and genetic influences, the subjects chosen for study were a cohort of twins discordant for diabetes who were free of complications of the disease at the time of study.

SUBJECTS AND METHODS

Subjects

We studied 15 identical-twin pairs discordant for insulin-dependent diabetes mellitus (one twin had diabetes and the other did not) who fulfilled the following inclusion criteria: age 20 to 65 years, white ethnicity, availability of both twins for study, absence of hypertension or microalbuminuria (albumin excretion rate >20 μg albumin/min in a timed overnight urine sample), and absence of treatment with prescribed antihypertensive drugs. The diabetic twin in each pair had been treated from the time of diagnosis (mean ± SD, 16 ± 10 years) with either highly purified porcine or human insulin.

We also studied 15 healthy control subjects who were individually matched with the discordant twin pairs for sex; in each individual case, control subjects were also matched with nondiabetic twins to within 5% with respect to age and body mass index. These control subjects were selected from a cohort of 100 subjects who were employees of local retail firms screened for the purpose of this study. None of the healthy subjects studied had any known

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family history of insulin-dependent diabetes, non–insulin-dependent diabetes, or hypertension or were from a multiple birth or took any drugs at the time of investigation.

All subjects were examined in the hospital between 10:00 AM and 4:00 PM. Subjects gave informed consent, and the study was approved by the Chelsea & Westminster Hospital Ethics Committee. Subjects underwent a thorough medical examination, and a full medical history was obtained from each. Information was requested regarding any family history of diabetes, hyperlipidemia, renal disease, or hypertension. Hypertension was defined according to World Health Organization criteria (blood pressure > 160/95 mm Hg).[7] Diabetes was excluded in all nondiabetic twins by a 75-g oral glucose tolerance test and also by a random whole-blood glucose assay at the time of testing. The healthy control subjects were accepted as demonstrating normal glucose tolerance if they had whole-blood fasting glucose concentrations less than 4.4 mmol/L, and less than 5.6 mmol/L if the sample was nonfasting.[8]

Fasting venous blood samples were drawn for biochemical analysis (Table 1), determination of sodium-lithium countertransport activity, and confirmation of monozygosity.[9] Timed urine samples were also collected from 10:00 PM to 8:00 AM during the night before the study. The urinary albumin excretion rate was assessed on this overnight sample; urinary albumin concentration was measured by rate nephelometry on a Beckman Array (Beckman, High Wycombe, Bucks, UK).

Characterization of the Sodium-lithium Countertransporter

Sodium-lithium countertransport activity was estimated using a method described previously.[4] Briefly, erythrocytes were incubated for 3 hours in lithium loading solution (LiCl 148 mmol/L, glucose 10 mmol/L, Tris-MOPS [pH 7.4 at 37°C] 10 mmol/L). Following incubation, erythrocytes were washed six times with a choline medium (choline chloride 139 mmol/L, $MgCl_2$ 1 mmol/L, glucose 10 mmol/L, ouabain 0.1 mmol/L, and Tris-MOPS [pH 7.4 at 4°C] 10 mmol/L).

Table 1. Clinical and Laboratory Characteristics of 15 Twin Pairs Discordant for Diabetes and a Matched Group of Healthy Controls

Characteristic	Controls	Twins	
		Nondiabetic	Diabetic
Gender (M/F)	10/5	10/5	10/5
Age (yr)	31.7 ± 8	30.5 ± 9	30.5 ± 9
Duration of diabetes (yr)	NA	NA	13 ± 5
BMI (kg/m²)	23.7 ± 2.1	23.6 ± 2.2	23.3 ± 2.1
Alcohol intake (U/wk)	4.0 (0-40)	3.5 (0-60)	4.5 (0-56)
SBP (mm Hg)	120 ± 16	122 ± 16	124 ± 17
DBP (mm Hg)	78 ± 11	77 ± 11	77 ± 13
Total serum cholesterol (mmol/L)	5.0 ± 1.0	5.1 ± 1.2	5.5 ± 1.1
Triglycerides (mmol/L)	1.5 ± 1.0	1.5 ± 1.2	1.7 ± 1.4
HDL cholesterol (mmol/L)	1.2 ± 0.2	1.2 ± 0.2	1.3 ± 0.3
LDL cholesterol (mmol/L)	3.0 ± 1.0	3.1 ± 1.0	3.3 ± 0.8
Blood glucose (mmol/L)	4.9 ± 1.1	5.3 ± 1.2	10.1 ± 5.2*
Hemoglobin A_{1c} (%)	6.0 ± 1.5	6.8 ± 1.6	10.1 ± 2.7*
Serum creatinine (μmol/L)	89.9 ± 8.2	89.6 ± 8.7	88.1 ± 7.9
Urinary albumin excretion rate (μg/min)	3.6 (1.5-12.0)	3.5 (1.9-11.2)	4.0 (2.1-14.7)

NOTE. Values are the mean ± SD, except for alcohol intake and albumin excretion rates, which are the median (range).

Abbreviations: SBP, systolic blood pressure; DBP, diastolic blood pressure; BMI, body mass index; HDL, high-density lipoprotein; LDL, low-density lipoprotein; NA, not applicable.

*$P < .05$: diabetic twins *v* nondiabetic twins and controls.

After the final washing, 0.2 mL of the erythrocyte pellet was used to estimate intracellular lithium concentration to ensure that the loading procedure had achieved sufficient intracellular lithium to saturate the internal lithium binding sites throughout the course of the experiment (intracellular lithium concentration, 9.4 ± 3.4 mmol/L). Aliquots of packed cells were added to either choline medium (as above) or sodium medium (NaCl 145 mmol/L, glucose 10 mmol/L, $MgCl_2$ 1 mmol/L, ouabain 0.1 mmol/L and Tris-MOPS [pH 7.4 at 37°C] 10 mmol/L) and incubated in a shaking water bath at 37°C. Samples were taken from each of the incubating solutions at 20, 40, and 60 minutes. After centrifugation of the incubation mixtures (3 minutes at 2,000 × *g*, 4°C), the supernatant was collected for determination of lithium content by atomic absorption spectrophotometry. Sodium-lithium countertransport activity was determined by subtracting the lithium efflux into the sodium-free media from that which occurred into the sodium-rich media, and expressed as millimoles of lithium released from 1 L erythrocytes/h.

Kinetic characteristics of the countertransporter were determined using a method we have described previously.[10] Lithium-loaded erythrocytes were prepared as above. Following the washing procedure, the cells were incubated in 10 different external media of varying sodium concentrations over the range of 0 to 150 mmol/L (0, 5, 10, 20, 40, 60, 80, 100, 125, and 150 mmol/L). Isotonicity was maintained in each case by inclusion of varying amounts of choline chloride plus $MgCl_2$ 1 mmol/L, glucose 10 mmol/L, ouabain 0.1 mmol/L, and Tris-MOPS [pH 7.4 at 37°C] 10 mmol/L. Incubations were performed and lithium efflux rates into each of 10 media were determined as already described for the traditional assay. The kinetic constants, k_{Na} and $\dot{V}_{max}$, were determined by plotting the flux rate in the different media against the flux rate/sodium concentration (Eadie-Hofstee plot). The data were fitted by linear regression, with k_{Na} values determined from the slope of the line and $\dot{V}_{max}$ from the intercept of the line with the y-axis. Correlation coefficients were at least .98 in all cases.

Kinetic characteristics of the sodium-lithium countertransporter were also measured in triplicate on fresh cells from 10 subjects on three different occasions over the 18 months of the study. The mean interassay and intraassay variations for both $\dot{V}_{max}$ and K_m were calculated from these results and found to be 9.8% ± 6.5% and 5.9% ± 3.9% and 11.1% ± 5.8% and 9.5% ± 5.5%, respectively. These levels of technical error and intraindividual variation are similar to the values we reported for sodium-lithium countertransport activity.[4]

Statistical Methods

Data analyses were performed using the SPSS statistical package (SPSS Inc, Chicago, IL). Results are expressed as the mean ± SD, median and range, or median and 95% confidence interval. Comparisons between discordant and concordant twin groups were made using paired Student's *t* tests when the data were normally distributed; Wilcoxon nonparametric tests were used when the data were not normally distributed. Comparisons between control and nondiabetic twin groups were made using unpaired Student's *t* tests when the data were normally distributed and Mann-Whitney nonparametric tests when they were not. Associations within twin pairs were assessed by product-moment correlation. In all statistical investigations, *P* less than .05 was considered significant.

In addition to standard statistical manipulation, it was possible in the discordant twin groups to calculate residual values of clinical and biochemical data by subtracting values obtained in nondiabetic twins from those of their corresponding diabetic twins. These data were correlated with the residual countertransport measurements of activity, $\dot{V}_{max}$ and k_{Na}, to see if any underlying metabolic differences between the twins were influencing sodium-lithium countertransport characteristics.

RESULTS

The clinical and laboratory characteristics of control, diabetic, and nondiabetic twin groups were similar, except for elevated random whole-blood glucose and glycated hemoglobin concentrations in the diabetic twin group (Table 1).

Kinetic characteristics of the sodium-lithium countertransporter are described in Table 2. Analysis of the data showed that $\dot{V}_{max}$ values conformed to a normal distribution pattern, whereas sodium-lithium countertransport activity and k_{Na} data did not.

With one exception (a subject with 0.418 mmol Li/L red blood cells [RBC] · h), sodium-lithium countertransport activities in control subjects (n = 15) were consistently less than the 0.400-mmol Li/L RBC · h cutoff value conventionally viewed as the normal upper limit for this measurement in healthy populations (Table 2).[5] Within the discordant twin population studied, seven individuals (five twins with diabetes and two separate nondiabetic twins) had sodium-lithium countertransport activity values greater than 0.400 mmol Li/L RBC · h (range, 0.460 to 0.683; n = 7). Comparison of countertransport activity between nondiabetic and diabetic twin groups failed to uncover any significant differences ($P = .30$, Wilcoxon). Similarly, there were no differences in countertransport activity between the nondiabetic twin group and controls ($P = .38$, Mann-Whitney). Furthermore, no associations were noted between residual activity values and residual data for any of the other clinical or laboratory charcteristics measured.

Comparison of $\dot{V}_{max}$ between nondiabetic and diabetic twin groups showed a significant elevation in the diabetic twins (0.515 ± 0.220 *v* 0.439 ± 0.229 mmol Li/L RBC · h, $P = .049$, paired *t* test). By contrast, no significant differences were noted between the nondiabetic twin group and controls ($P = .15$, unpaired *t* test). Comparison of k_{Na} between nondiabetic and diabetic twin groups found no significant differences in k_{Na} ($P = .42$, Wilcoxon). Similarly, there were no differences in k_{Na} between nondiabetic twins and controls ($P = .14$, Mann-Whitney nonparametric test; Table 2). Neither the residual data for $\dot{V}_{max}$ nor k_{Na} showed any association with the residual data for any of the other clinical or laboratory characteristics measured.

When intertwin correlations (between twin groups) were examined, all three parameters describing the behavior of the sodium-lithium countertransporter showed significant intertwin correlations (activity, $r = .51$, $P = .04$; $\dot{V}_{max}$, $r = .82$, $P = .001$; k_{Na}, $r = .76$, $P = .001$). The scatter of $\dot{V}_{max}$ values showed that 13 of 15 measured points were to the left of the line of identity (Fig 1). The range of intertwin residual data for countertransport activity (−0.212 to 0.391 mmol Li/L RBC · h) was similar to that of the residual $\dot{V}_{max}$ data (−0.211 to 0.426 mmol Li/L RBC · h). However, overall distribution characteristics for the residual values were strikingly different. In the case of residual sodium-lithium countertransport activity values, the shape of the distribution was flattened (platykurtic), with a kurtosis value of −0.28 (interquartile range for the 25th to 75th percentile, −0.096 to 0.17 mmol Li/L RBC · h). By contrast, $\dot{V}_{max}$ residual values showed a diametrically opposite shape for the distribution curve, being peaked (leptokurtic) with a kurtosis value of +3.5 (interquartile range, 0.024 to 0.123 mmol Li/L RBC · h).

DISCUSSION

Controversy surrounds quantification of the expression of erythrocyte sodium-lithium countertransport activity in the diabetic state. A number of initial reports showed that countertransport activity was elevated in diabetic patients with nephropathy compared with nonnephropathic diabetic subjects.[1,2] Several subsequent reports claimed that sodium-lithium countertransport activity was elevated in diabetes even in the absence of nephropathy.[4,11,12] Part of the reason for this apparent contradiction may be the failure to account for either the influence of the metabolic disturbance associated with diabetes or the complex nature of the countertransporter itself.

Hypertension and renal damage are common secondary complications of insulin-dependent diabetes, and both conditions have been linked to abnormal sodium-lithium countertransport activity.[1,2,13] The diabetic twins included in the present study were screened to eliminate subjects who were either overtly hypertensive, on antihypertensive therapy, or showing any evidence of nephropathy. By studying identical twins discordant for diabetes, we hoped to remove the confounding effect of genetic influences, thereby isolating the effect of diabetes per se on the behavior of this membrane transporter. Because the environment also can have significant effects on expression of countertransport activity,[3,5] subjects included in our control group were closely matched with the twin pairs.

The median (with range) and pattern of distribution of sodium-lithium countertransport activities in the study groups were similar to those seen in larger cohorts of discordant identical twins that we have reported elsewhere.[4] As in our earlier study,[4] the disease state itself was found to have no influence on sodium-lithium countertransport activity. However, by contrast to our previous findings, no significant differences were noted between the control

Table 2. Kinetic Characteristics of the Sodium-Lithium Countertransporter in 15 Twin Pairs Discordant for Diabetes and a Matched Group of Healthy Controls

Characteristic	Controls (n = 15)	Twins	
		Nondiabetic (n = 15)	Diabetic (n = 15)
SLC activity (mmol Li/L RBC · h)	0.198 (0.071-0.418)	0.199 (0.037-0.683)	0.285 (0.033-0.620)
$\dot{V}_{max}$ (mmol Li/L RBC · h)	0.388 ± 0.130	0.439 ± 0.229	0.515 ± 0.220*
K_{Na} (mmol)	90.5 (54.2-165.2)	110.0 (44.2-240.6)	103.7 (15.6-180.5)

NOTE. For sodium-lithium countertransport activity and k_{Na}, values are the median (range); $\dot{V}_{max}$ values are the mean ± SD.
*$P < .05$, diabetic *v* nondiabetic twins.

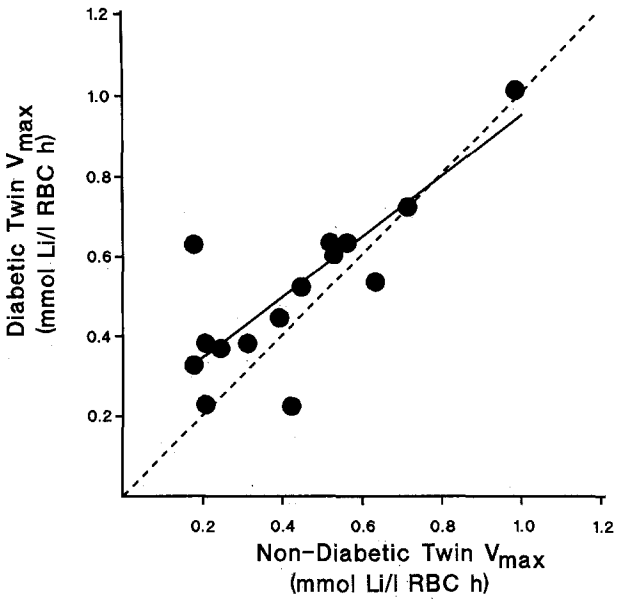

Fig 1. Comparison of values for $\dot{V}_{max}$ obtained from identical twins (n = 15) discordant for insulin-dependent diabetes mellitus. (- - -) Line of identity; (—) line of regression.

subjects and twin groups. This may simply reflect the smaller number of twins included in the present study. There was a trend toward higher activity values in the twin groups. One subject in the present control group had a sodium-lithium countertransport activity greater than 0.400 mmol Li/L RBC · h, whereas two nondiabetic and five diabetic twin subjects exceeded this threshold value. The degree of correlation between sodium-lithium countertransport activities of the twin groups was similar to that seen for other discordant diabetic twins we have studied,[4] but was not as strong as that observed between either healthy identical twin pairs or identical twin pairs concordant for diabetes.[14,15] This suggests that some additional factor(s) may be influencing countertransport activity.

Expression of countertransport activity itself is inherently flawed. In many publications, sodium-lithium countertransport activity has been viewed as a biochemical constant rather than a lithium ion efflux rate determined under specific experimental conditions. Indeed, the considerable variation between laboratories in the way sodium-lithium countertransport activity has been measured adds further to the difficulty in comparing data from different studies. When residual data for sodium-lithium countertransport activity and $\dot{V}_{max}$ were compared, the values were in similar ranges. However, $\dot{V}_{max}$ values were more closely associated between twins than activity values, as demonstrated by the leptokurtic versus platykurtic distributions of the residual data. This may reflect the stricter dependence of countertransport activity on the conditions under which it is measured, as compared with determination of $\dot{V}_{max}$, which behaves as a biochemical constant.

Paired *t* tests between diabetic and nondiabetic twin groups uncovered an elevation in the $\dot{V}_{max}$ of the twins. This observation is in agreement with other studies showing that insulin-dependent diabetes is accompanied by an increase in $\dot{V}_{max}$.[16]

It has been suggested that some degree of the changes in behavior of the countertransporter in diabetes can be ascribed to alterations in plasma lipids. However, such alterations could not fully explain the changes in the countertransporter, since diabetic patients investigated by Rutherford et al[16] who had elevated $\dot{V}_{max}$ values also had similar plasma lipid profiles compared with healthy control subjects. In the present study, both twin groups and controls demonstrated equivalent plasma lipid profiles, and so, once again, differences in lipid profiles are unlikely to explain the elevation in $\dot{V}_{max}$. Furthermore, our failure to uncover any associations between the residual $\dot{V}_{max}$ values and residual data for the lipid measurements makes it improbable that either plasma triglyceride or high-density or low-density lipoprotein cholesterol is responsible for the alteration in $\dot{V}_{max}$ associated with diabetes.

The present study found no evidence for an association between diabetes and alterations in the k_{Na} of the countertransporter. This would agree with other studies that have only shown changes in k_{Na} when diabetes is accompanied by hypertension or nephropathy.[17-19]

When intertwin correlations for both kinetic constants of the countertransporter were determined, there were high degrees of association for both k_{Na} and $\dot{V}_{max}$. This is likely to reflect the strong genetic influences on the countertransporter that have been noted for countertransport activity on a number of previous occasions[20,21]; they have only been observed once previously for k_{Na}.[22] A genetic influence on $\dot{V}_{max}$, which is also sensitive to environmental factors,[23] has not been previously described. No relationships emerged between the residual kinetic data and the data for any of the clinical or laboratory patient characteristics.

Failure to consider the complex nature of the activity measurement is likely to be partly responsible for the earlier confusion with regard to the effect of diabetes on the countertransporter, since experimental conditions varied between studies and individual kinetic components were not measured. The associations between the twins in this study with respect to $\dot{V}_{max}$ and k_{Na} may indicate a genetic influence on both constants of the countertransporter. $\dot{V}_{max}$ may also be sensitive to certain as yet unidentified environmental factors.

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