Na/Li Countertransport Abnormalities in Type 1 Diabetes With and Without Nephropathy Are Familial

**Paul A. Mead, MBBS**

**Robert Wilkinson, MD**

**Trevor H. Thomas, PhD**

**OBJECTIVE** — To determine whether there is a familial abnormality in erythrocyte Na/Li countertransport (CT) kinetics in the approximate one-third of type 1 diabetic patients that succumb to a familial predisposition to nephropathy.

**RESEARCH DESIGN AND METHODS** — Erythrocyte Na/Li CT kinetics were measured in nondiabetic first-degree relatives of type 1 diabetic patients with nephropathy (DNrels) (*n* = 32) or without nephropathy (DCrels) (*n* = 22) and normal control subjects (*n* = 25).

**RESULTS** — Increases in outside-site Na ion association rate constant and turnover rate of Na/Li countertransport (CT) in DNrels caused increases in V$_\text{max}$/K$_m$ and V$_\text{max}$, respectively. Thiol alkylation with N-ethylmaleimide (NEM) modifies these kinetic parameters abnormally in nephropathy. With Na ions at the outside site of the transporter, thiol alkylation causes a large decrease in V$_\text{max}$; but in their absence, V$_\text{max}$ is decreased in normal control subjects, unchanged in DCrels, or increased in DNrels. The relationship between V$_\text{max}$ values after thiol alkylation with or without Na ions was different in DNrels (*P < 0.001*). Kinetic parameters with and without thiol alkylation identified 60% of DNrels and 20% of DCrels as abnormal. The single-flux rate assay of Na/Li CT did not give this discrimination, and its use may cause discrepancy between studies.

**CONCLUSIONS** — Clinically normal untreated DNrels have the same abnormality in Na/Li CT as the affected patients. DNrels had a metabolic syndrome with increased BMI and plasma triglycerides, but no elevation in blood pressure. Na/Li CT can detect those type 1 diabetic patients at risk of nephropathy who have a familial abnormality in a membrane thiol protein.

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Nephropathy is a major complication of type 1 diabetes; once established, the prognosis is poor because of the accompanying cardiovascular disease. Presently, the earliest diagnosis relies on the appearance of microalbuminuria; but by this stage, significant kidney damage has already occurred. Approximately one-third of type 1 diabetic patients are at risk of developing nephropathy, and this risk is familial (1).

Erythrocyte Na/Li countertransport (CT), measured as a lithium efflux rate at 140 mmol/l sodium, has been shown to be abnormal in type 1 diabetic patients with nephropathy (2–5). Because Na/Li CT has a strong inherited component (6,7), it is possible that this could be a marker for the susceptibility to nephropathy. Three studies showing significant correlations between Na/Li CT flux rate in diabetic patients with nephropathy and their parents provide evidence in support of this hypothesis (8–10).

However, in direct contradiction to these findings, other studies found that Na/Li CT was increased in all diabetic patients and that it was not related to nephropathy (11,12). Furthermore, another study showed that there was no difference in Na/Li CT between parents of type 1 diabetic patients with or without nephropathy (13). The importance of predicting patients at risk of nephropathy makes it worthwhile to resolve this discrepancy. A recent study found a very significant risk association of Na/Li CT with the development of microalbuminuria in type 1 diabetes, but the specificity for individual patients was low (14).

Simple ion flux assays, as used in previous clinical studies, have been criticized because they offer a poor level of information and are liable to confusion from confounding effects (15). We suggest that this is the reason for the discrepancy. Recently, we have shown that in groups of type 1 diabetic patients with and without nephropathy, there was no significant difference in Na/Li CT flux rate at 140 mmol/l Na, but there were marked differences in Na/Li CT kinetic parameters (16). As we have previously discussed, the Michaelis constant for external Na ($K_m[So]$) of Na/Li CT does not reliably indicate ion affinity (17,18). This is because the ion translocation rate constant of Na/Li CT is large compared with its Na ion association rate constant (19); therefore, both of these rate constants affect $K_m[So]$. Thus, a decrease in the ion translocation rate constant (transporter turnover) causes a decrease in both V$_\text{max}$ and $K_m[So]$, but V$_\text{max}$/K$_m$ will be independent of V$_\text{max}$. In this situation, V$_\text{max}$/K$_m[So]$ is a more reliable indication of the ion association rate constant than $K_m$, but V$_\text{max}$ may also increase because of the increased numbers of transporters. Therefore, V$_\text{max}$/
RESEARCH DESIGN AND METHODS

Patients and normal control subjects
A total of 25 normal control subjects (12 men and 13 women) from the University of Newcastle-upon-Tyne, health service staff, and the local community participated in the study. They had normal blood pressure (<140/90 mmHg), plasma glucose (fasting <6.0, random <8.0 mmol/l), and serum lipids (cholesterol <6.2, triglycerides <2.0 mmol/l), and no known family history of diabetes, hypertension, or hyperlipidemia. Also, 22 nondiabetic first-degree relatives of type 1 diabetic patients (DCrel) (11 male and 11 female: 11 offspring and 11 siblings) with a duration of diabetes >25 years; no significant eye disease (normal fundoscopy or background retinopathy only); and normal blood pressure, serum creatinine, and urinary albumin excretion were included in the study. In addition, 32 nondiabetic relatives of type 1 diabetic patients with nephropathy (DNrel) (16 male and 16 female: 14 offspring and 18 siblings), with a duration of diabetes <20 years, a history of hypertension, and variations from elevated urinary albumin excretion to end-stage renal disease participated in the study.

The Joint Ethics Committee of Newcastle Health Authority and Newcastle University approved the study, and subjects gave their informed consent.

Na/Li CT assay
The method for the Na/Li CT assay was similar to that previously described (18).

<p>| Table 1—Kinetic parameters of Na/Li CT in normal control subjects and first-degree relatives of diabetic patients (DNrel) and without (DCrel) nephropathy |
|-----------------|-----------------|-----------------|-----------------|-----------------|</p>
<table>
<thead>
<tr>
<th></th>
<th>Normal control subjects</th>
<th>DCrels</th>
<th>DNrels</th>
<th>ANOVA P</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>25</td>
<td>22</td>
<td>32</td>
<td></td>
</tr>
<tr>
<td>Na/Li CT (flux rate at 140 mmol/l Na)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vmax</td>
<td>0.22 ± 0.01</td>
<td>0.23 ± 0.03</td>
<td>0.29 ± 0.02</td>
<td>0.01</td>
</tr>
<tr>
<td>Ken</td>
<td>0.37 ± 0.03</td>
<td>0.34 ± 0.03</td>
<td>0.43 ± 0.02</td>
<td>0.01</td>
</tr>
<tr>
<td>Vmax/Ken</td>
<td>0.98 ± 6</td>
<td>72 ± 5†</td>
<td>73 ± 6†</td>
<td>0.001</td>
</tr>
</tbody>
</table>

Data are means ± SEM. Na/Li CT and Vmax are expressed as mmol L/h per liter erythrocyte. Ken is expressed as mmol Na/l. *P < 0.05 vs. controls; †P < 0.05 vs. normal control subjects; ‡P < 0.05 vs. DCrel; §P < 0.001 vs. DNrel. ANOVA P < 0.001 NEM vs. no treatment.

Erythrocytes from Li-heparinized (125 IU/10 ml) venous blood were incubated in Li-loading solution (140 mmol/l LiCl, 10 mmol/l Li2CO3, 10 mmol/l glucose, and 10 mmol/l Tris-MOPS, pH 7.5, 290 ± 2 mosmol/kg) for 1.5 h. Erythrocytes were then washed in choline medium (139 mmol/l choline chloride, 1 mmol/l MgCl2, 10 mmol/l glucose, and 10 mmol/l Tris-MOPS, pH 7.4, 290 ± 2 mosmol/kg) and incubated at a packed cell volume between 3 and 6% in choline- ouabain medium (10−4 mol/l ouabain) or medium with a range of Na concentrations (20–150 mmol/l) made by replacing choline with Na. Samples were taken up to 90 min incubation at 37°C for Li measurement using a Perkin-Elmer 3110 atomic absorption spectrometer. Each erythrocyte contained 9.0 ± 1.0 mmol Li. Osmolality was measured using a Camlab osmometer.

Kinetic parameters of Na/Li CT activity
Flux rate = Vmax − Ken(So) × flux rate/ [Na]e gave Ken(So) from the slope, Vmax from the intercept on the y-axis, and Vmax/ken from the intercept on the x-axis of the plot of flux rate versus flux rate/ [Na]e.

Thiol group alkylation with NEM
Erythrocytes (0.5 ml) washed free of external Na with choline medium were suspended in 3 ml choline or Na medium and excess NEM (3 μmol in 100 μl choline medium) was added (20) to the prewarmed suspension and incubated at 37°C for 100 s. The reaction was stopped with a fivefold excess of mercaptoethanol in choline medium. Native erythrocytes suspended in choline medium were treated with mercaptoethanol only. Erythrocytes were washed in choline medium, and Na/Li CT was assayed as previously described.

Statistics
Values were normally distributed, except for urine albumin excretion, and with this exception were presented as mean and SE of the mean. Differences among all groups were assessed using analysis of variance (ANOVA) and, subsequently, Student’s t test among individual groups. Differences within groups were assessed using the paired Student’s t test. In regression analysis between kinetic parameters, the mean line for y on x and x on y was used because there was error in both x and y values.

RESULTS
Na/Li CT in untreated erythrocytes
DNrels were abnormal for each of the Na/Li CT kinetic parameters. Both Vmax and Vmax/ken were increased, and Ken was decreased compared with normal control subjects (Table 1). DCrels had a Vmax similar to that of normal control subjects and
lower than that of DNrels. However, $K_m$ of DCrels was similar to that of DNrels and lower than that of normal control subjects, and $V_{\text{max}}/K_m$ was increased, although it was still lower than that of DNrels. The Na/Li CT flux rate at 140 mmol/l Na was increased in DNrels (Table 1), and 37% of DNrels and 18% of DCrels had values greater than the 95th percentile value for normal control subjects.

The effects of NEM on Na/Li CT kinetics

NEM reaction in Na medium caused a large decrease in $V_{\text{max}}$ in all groups, but $V_{\text{max}}$ remained elevated in DNrels (Table 1). This value, together with $V_{\text{max}}/K_m$ in untreated erythrocytes, was a good discriminator of DNrels (Fig. 1). The two kinetic parameters had a similar relationship in each of the three groups (normal control subjects $r = 0.4$, $P = 0.05$; DCrels $r = 0.82$, $P < 0.001$; and DNrels $r = 0.71$, $P < 0.001$). The 95th percentile values for normal control subjects gave discriminating values similar to those previously used for diabetic patients (16) and showed that 56% of DNrels and 18% of DCrels were outside the normal control values.

NEM reaction in choline medium caused a slight decrease in $V_{\text{max}}$ in normal control subjects; there was no change in DCrels and an increase in DNrels (Table 1). These differences in response among the three groups were statistically significant (ANOVA $P < 0.001$). There were decreases in $K_m$ in both normal control subjects and DCrels, but not in DNrels; however, $V_{\text{max}}/K_m$ increased in all three groups. The $V_{\text{max}}$ values remaining after NEM reaction in Na or choline medium were positively correlated in normal control subjects (slope 0.239, SEM 0.063, $P < 0.001$), DCrels (slope 0.372, SEM 0.027, $P < 0.001$), and DNrels (slope 0.195, SEM 0.035, $P < 0.001$), although the changes were in different directions in DNrels. The slopes for DCrels and DNrels were significantly different ($P < 0.001$) (Fig. 2). In addition, by using the 95% range for normal control values (Fig. 2), these kinetic parameters provided an effective discrimination of DNrels, with 63% outside the normal control range for both parameters. How-

![Figure 1](image1.png)

**Figure 1**—The relationship between two kinetic parameters of Na/Li CT: $V_{\text{max}}$ after reaction of erythrocytes with NEM in Na medium and $V_{\text{max}}/K_m$ in untreated erythrocytes in normal control subjects (○), DCrels (▲), and DNrels (●). The 95% lines for normal control subjects for each parameter are shown (-----).

![Figure 2](image2.png)

**Figure 2**—The relationship between $V_{\text{max}}$ of Na/Li CT after reaction of erythrocytes with NEM in either Na medium or choline medium in erythrocytes from normal control subjects (○, -----), DCrels (▲, --- ---), and DNrels (●, ---- - - - -). The slopes of the regression lines for DNrels and DCrels are significantly different ($P < 0.001$). The 95% lines for normal control subjects for each parameter are shown (-----).
ever, 18% of DCrels were also outside this range.

**Clinical features associated with a family history of diabetic nephropathy**

DNrels had significantly higher BMI and plasma triglycerides than DCrels, but normal blood pressure (Table 2). There was no indication that abnormal Na/Li CT within DNrels was associated with raised blood pressure, because those relatives with Na/Li CT parameters clearly outside the normal range, according to the discriminators previously described, had a mean blood pressure of 124/75 mmHg (±4/4) (n = 16).

Multiple regression of plasma triglycerides, BMI, and mean arterial pressure with the kinetic parameters of Na/Li CT showed a strong positive correlation of Vmax with plasma triglycerides in all relatives (P < 0.001 and in DNrels (P < 0.001) and DCrels (P < 0.05). Vmax also had a weak negative association with BMI in DNrels (P = 0.06), but a negative association with mean arterial pressure in DCrels (P < 0.003). Vmax/Km was similarly correlated with plasma triglycerides in all relatives (P < 0.002) and in DNrels (P < 0.02), but there were no significant correlations with Km. There were also no significant correlations with the changes in kinetic parameters with NEM reaction, although the Vmax remaining after NEM reaction remained correlated with plasma triglycerides alone in all relatives (P < 0.005), in DCrels (P < 0.001), and in DNrels (P < 0.02). The relatives outside the 95% normal control range in Figs. 1 and 2 had no significant differences in plasma triglycerides, BMI, or blood pressure compared with the rest of their group.

**Table 2—Clinical measurements in DNrels and DCrels**

<table>
<thead>
<tr>
<th></th>
<th>DCrels</th>
<th>DNrels</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>39 ± 3</td>
<td>41 ± 2</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>23.6 ± 0.7</td>
<td>29.2 ± 1.0*</td>
</tr>
<tr>
<td>Blood pressure (mmHg)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Systolic</td>
<td>131 ± 3</td>
<td>129 ± 3</td>
</tr>
<tr>
<td>Diastolic</td>
<td>79 ± 2</td>
<td>77 ± 3</td>
</tr>
<tr>
<td>Triglycerides (mmol/l)</td>
<td>1.43 ± 0.17</td>
<td>1.97 ± 0.17†</td>
</tr>
<tr>
<td>Cholesterol (mmol/l)</td>
<td>5.08 ± 0.21</td>
<td>5.29 ± 0.15</td>
</tr>
</tbody>
</table>

Data are means ± SE. *P < 0.001 and †P < 0.01 vs. DCrel.

**CONCLUSIONS**

**First-degree relatives have abnormal Na/Li CT**

The present study shows that the first-degree relatives of diabetic patients with nephropathy have the same abnormality in their Na/Li CT kinetics as diabetic patients with nephropathy. These relatives have no clinically detectable abnormality and are without any drug treatment, which excludes these factors as causes for abnormal Na/Li CT kinetics. This indicates a major genetic factor in the abnormality of Na/Li CT associated with diabetic nephropathy. The majority of the relatives have values for the key kinetic parameters outside the 95th percentile range for normal control subjects. This suggests that the type 1 diabetic patients at risk of nephropathy who have the abnormality in Na/Li CT can be identified very early in (if not before) the development of diabetes.

The kinetic parameters of Na/Li CT that distinguish the relatives of diabetic patients with nephropathy are the Vmax/Km ratio and the Vmax values after thiol alkylation in the presence or absence of Na ions (Fig. 1). Figure 1 uses two parameters that have the greatest mean differences between DNrels and the other groups and provides a discriminator that we have shown to be effective in diabetic patients with nephropathy (16). Discrimination with this plot was clearer for DNrels than for diabetic patients with nephropathy (16). Thus, the abnormal Na/Li CT is highly prevalent among first-degree relatives of affected patients and may be expected from the high concordance for nephropathy in diabetic siblings (21). A relation between Vmax/Km of Na/Li CT in IgA nephropathy patients and their first-degree relatives has also been observed (22).

The distribution of values in DNrels is consistent with either a continuous distribution overlapping into the normal range or two populations of DNrels, one having familial susceptibility for diabetic nephropathy associated with abnormal Na/Li CT.

There are also abnormalities in Na/Li CT in DCrels that suggest the same factors are affected as in DNrels, but to a much lesser extent. This implies that the function of these membrane proteins is associated with a familial risk of type 1 diabetes. An association between abnormal Na/Li CT and type 1 diabetes should be expected, because if 60% of diabetic patients with nephropathy have abnormal Na/Li CT kinetics and if 30% of type 1 diabetic patients will develop nephropathy, then 20% of type 1 diabetic patients have abnormal Na/Li CT kinetics, which is a much greater proportion than that seen in the general population. These observations in clinically normal first-degree relatives clearly show an association between abnormal Na/Li CT and an inherited susceptibility factor rather than the disease itself.

**Two abnormalities in Na/Li CT**

The difference in Na/Li CT kinetic parameters between DNrels and normal control subjects strongly implicates thiol proteins in the abnormality. The two abnormalities are 1) increased Na ion association rate constant (Vmax/Km) and 2) abnormal control of transporter turnover (Vmax). The former DNrel abnormality can be mimicked by NEM alkylation of thiol groups in normal control or DCrel erythrocytes. In contrast, NEM reaction accentuates the differences in abnormal control of transporter turnover (Vmax) between DNrels and the other groups.

Two types of thiol that modify Na/Li CT kinetics can be distinguished, because NEM only reacts with one thiol in choline medium. The second thiol only reacts in the presence of Na ions. In normal control erythrocytes, the former reaction increases the external Na association rate constant (increase in Vmax/Km with a decrease in Km), hence mimicking the high Vmax/Km in DNrels. The abnormal increase in turnover rate (increase in Vmax) with this reaction in DNrels makes Km unreliable, as previously described. The
latter reaction causes a decrease in turnover rate (decrease in $V_{\text{max}}$).

Alkylation of either of these thiol groups leaves $V_{\text{max}}$ higher in DNrels than in the other groups, and this is the clearest abnormality from DCrels. It is not clear whether the two thiols are on the same protein. Therefore, the relationship between their effects on $V_{\text{max}}$ was examined (Fig. 2), and the correlation between the $V_{\text{max}}$ values after alkylation of either thiol group in each of the three groups of subjects supports a single protein, or at least two closely associated proteins. This correlation exists despite large decreases in $V_{\text{max}}$ caused by alkylation of the Na-sensitive thiol and the paradoxical increases in $V_{\text{max}}$ caused by alkylation of the Na-insensitive thiol in DNrels. However, the relationship between the changes caused by alkylation of these two thiols is significantly different in DNrels, which suggests that the thiol protein(s) has an abnormal association in the complex that modulates Na/Li CT kinetics. We have suggested that a 33-kDa cytoskeletal protein is responsible (23), but this protein could be abnormal, or its association with Na/Li CT could be altered by other factors.

Clinical associations with abnormal Na/Li CT in relatives

There was evidence for a metabolic type of syndrome in DNrels with increased BMI and plasma triglycerides, which are commonly found in essential hypertension patients with normal Na/Li CT. Multiple regression showed that this metabolic component was associated with $V_{\text{max}}$, probably due to increased transporter numbers, because $V_{\text{max}}/K_m$ had a similar association, but $K_m$ was not related to the metabolic factors. Na/Li CT kinetics in DNrels were very similar to those in essential hypertension patients (18): increased $V_{\text{max}}/K_m$ at baseline and NEM reaction in the absence of Na failed to cause the normal combination of a decrease in $K_m$ and an increase in $V_{\text{max}}/K_m$, indicating an increase in Na association rate constant, rather, it caused an increase in $V_{\text{max}}$, indicating an increased turnover rate. However, there was no indication of raised blood pressure levels in DNrels, although diabetic nephropathy has been associated with raised blood pressure in previous studies. In fact, the blood pressures in DNrels and DCrels were the same and similar to those in previous studies that refuted the association (13). The issue of familial hypertension in diabetic nephropathy is unresolved and is hampered by the lack of a plausible mechanism linking abnormal Na/Li CT to either hypertension or diabetic nephropathy. Thus, in the study population, ~60% of DNrels have a familial abnormality in a membrane thiol protein that leads to abnormal Na/Li CT kinetics. The latter abnormality is the same as that previously observed in patients with diabetic nephropathy. This suggests that the abnormality precedes the onset of nephropathy and should allow patients at risk of nephropathy to be identified at any time before or after the onset of diabetes.

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References
20. Thomas TH, West IC, Wilkinson R: Mod-
Diabetic nephropathy and Na/Li countertransport

