Thiol Protein Defect in Sodium-Lithium Countertransport in Subset of Essential Hypertension

Paul Mead, Robert Wilkinson, Trevor H. Thomas

Abstract—There is probably a heterogeneous etiology for essential hypertension (EHT), and abnormal erythrocyte sodium-lithium countertransport (Na/Li CT) is common in a subgroup of patients with a strong family history of hypertension and cardiovascular disease (EHT-FH patients). The aim of this study was to test the hypothesis that altering a membrane thiol protein could mimic the abnormal Na/Li CT observed in the patients and that a more refined understanding of the mechanism of abnormal Na/Li CT would facilitate a clearer identification of a subgroup of patients with a homogeneous biochemical abnormality. Na/Li CT kinetics were determined in untreated erythrocytes and after thiol group alkylation with N-ethylmaleimide (NEM). Compared with normal control erythrocytes, untreated erythrocytes from EHT-FH patients had a low $K_m$ of Na/Li CT, with a high ratio of maximum velocity to $K_m$. This kinetic pattern was reproduced in normal erythrocytes by treatment with NEM in sodium-free medium. The same treatment in EHT-FH erythrocytes caused a markedly abnormal effect with an increase in maximum velocity, indicating an increase in transporter turnover in contrast to the increase in sodium affinity seen in normal control erythrocytes. Frequency distributions of these kinetic changes showed a subgroup of $\approx75\%$ of EHT-FH patients with abnormal kinetic changes with NEM. Therefore, the key Na/Li CT thiol group that is very reactive to NEM and causes the abnormal Na/Li CT in a subgroup of hypertensive patients may be a useful intermediate phenotype for a disease group within the syndrome of EHT. The single flux assay of Na/Li CT at 140 mmol/L sodium poorly discriminates this group. Identification of the thiol protein involved may lead to a molecular explanation of the altered membrane function in this subgroup of patients.

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Key Words: erythrocytes ■ hypertension, essential ■ kinetics ■ sodium-lithium countertransport

Essential hypertension (EHT) is common in developed Western societies and is a major risk factor for cardiovascular disease. EHT is not a single disease, and because blood pressure is normally distributed in populations, EHT is difficult to define even as a clinical syndrome and has a wide range of severity. Current guidelines are therefore to treat all patients with sustained blood pressure above a particular level, which varies in different countries. Because it is not possible to predict which patients will develop a complication, many treated patients would never have suffered a complication if left untreated. A trial of the treatment of mild hypertension (diastolic blood pressure up to 110 mm Hg) found that 850 patient years of treatment prevented one stroke and did not alter the occurrence of myocardial infarction.

Approximately 95% of hypertensive patients are classified as having EHT. It seems unlikely that this large group will be homogeneous, and there are probably multiple etiologic factors. However, in patients with relatively severe hypertension and with a strong family history of hypertension and a high incidence of cardiovascular disease, a marker in the erythrocyte membrane detected as a sodium-lithium countertransporter has altered activity. In vivo, in the absence of lithium, this transporter must give equimolar sodium-sodium exchange, and its physiological role is obscure.

The initial measurement of sodium-lithium countertransport (Na/Li CT) was as a lithium flux rate from lithium-loaded erythrocytes with 140 mmol/L external sodium. An activity $>0.4$ mmol/h×L red blood cell (rbc) was considered abnormal, but although this was initially promising, a recent meta-analysis of many studies has shown that discrimination between EHT and normal is poor. However, Na/Li CT activity is increased not only by the familial factor related to EHT but also by metabolic factors associated with dyslipidemia, and values $>0.4$ have been much less common in successive studies, even from the same laboratory. This is probably due to improved management of plasma lipids. The use of simple ion flux rates in clinical studies has been criticized as giving a poor level of information and being susceptible to confounding factors. Thus, changes in maximum velocity ($V_{max}$) or ion binding events ($K_m$ or $V_{max}/K_m$) cannot be discriminated and may be missed. Kinetic studies of Na/Li CT showed that whereas hyperlipidemia increased $V_{max}$, the defect in EHT was a low $K_m$ for sodium at the external...
site. However, a molecular explanation for this abnormality is still awaited.

The sensitivity of Na/Li CT to thiol-alkylating agents such as N-ethylmaleimide (NEM) is the only information available regarding a protein involved with the transporter. It is clear that at least 2 types of thiol group modify the kinetics of Na/Li CT, and these can be shown by their unusual reactivity characteristics with NEM compared with most erythrocyte thiols. One of these thiols reacts only in sodium or lithium medium and causes a large decrease in activity at 140 mmol/L sodium, with lower Vₘₐₓ Kₘ and Vₘₐₓ/Kₘ. This abolishes the difference in Na/Li CT between normal control subjects (NCs) and patients with EHT. Thus, a thiol protein is strongly implicated in the abnormality, but this thiol reaction cannot explain the increased activity at 140 mmol/L sodium with the decreased Kₘ and increased Vₘₐₓ/Kₘ that are found in EHT. However, a second thiol group that reacts rapidly with NEM in the absence of sodium or lithium ions in normal erythrocytes causes a decrease in Kₘ for external sodium with little effect on Vₘₐₓ. This gives a kinetic pattern very similar to that seen in untreated erythrocytes from patients with EHT. This thiol group has a very acidic pKₐ and the specificity of the NEM reaction for the effect of this thiol on Na/Li CT can be shown by carrying out the reaction rapidly at pH 6, a level at which most erythrocyte thiols are reduced and their reaction with NEM is greatly reduced.14

Na/Li CT Assay

The method used was similar to that described previously. Venous blood was collected into polypropylene tubes containing lithium heparin (125 IU/10 mL blood). The erythrocytes were incubated in lithium-loading solution (in mmol/L: LiCl 140, Li₂CO₃ 10, glucose 10, and Tris-MOPS 10 [pH 7.5], at 290±2 mOsm/kg) for 1.5 hours. Erythrocytes were then washed 3 times in choline medium. After the final washing, the cells were suspended in choline medium to a volume of 4.5 mL. The packed cell volume of the erythrocytes was measured using a microhematocrit, and 200 µL aliquots of the red cell suspension were incubated in 1.5 mL choline-ouabain medium (in mmol/L: choline chloride 139, MgCl₂ 1, glucose 10, and Tris-MOPS 10 [pH 7.4], at 290±2 mOsm/kg containing 10⁻⁴ mol/L ouabain) or 1.5 mL of medium with a range of sodium concentrations (20 to 150 mmol/L) made by mixing choline-ouabain medium with sodium-ouabain medium (in mmol/L: NaCl 150, MgCl₂ 1, glucose 10, and Tris-MOPS 10 [pH 7.4], at 290±2 mOsm/kg containing 10⁻⁴ mol/L ouabain). Samples were taken during incubation for up to 90 minutes at 37°C. After centrifugation of incubation mixtures at 2000g for 3 minutes, 1 mL supernatant was mixed with 1 mL deionized water containing 0.1% Triton X-100, and the lithium content was measured by use of a Perkin-Elmer 3110 atomic absorption spectrometer with incubation media blanks. Erythrocytes after lithium loading and washing contained 8.5±1.0 mmol Li/L rbc. Osmolality was measured with a Camlab osmometer and adjusted as appropriate.

Thiol Group Alkylation with NEM

Erythrocytes were washed free of external sodium with choline medium. For reaction with NEM, erythrocytes (0.5 mL) were suspended in 3 mL of choline medium. NEM (3 µmol in 100 µL choline medium) was added to the prewarmed suspension and incubated at 37°C for 100 seconds. The reaction was stopped by the addition of a 5-fold excess of mercaptoethanol in choline medium. Untreated erythrocytes were suspended in choline medium and treated with mercaptoethanol. All erythrocytes were then washed in choline medium, and Na/Li CT was assayed as described above.

Kinetic Parameters of Na/Li CT Activity

To calculate the Michaelis-Menten constant for external sodium, given as Kₘ(So), and Vₘₐₓ of Na/Li CT, the standard equation is as follows:

\[ \text{flux rate} = V_{\text{max}} \times [\text{Na}^+]e/[\text{Na}^+]e + K_m(\text{So}) \]

This standard equation relating these parameters to the flux rate and the sodium concentration of the medium ([Na]ₑ) was rearranged by the Eadie-Hofstee method as follows:

\[ \text{flux rate} = V_{\text{max}} - K_m(\text{So}) \times \text{flux rate/}[\text{Na}^+]e \]

The flux rate was plotted against the flux rate/[Na]ₑ. The Kₘ(So) was determined from the slope; Vₘₐₓ, from the intercept on the y-axis; and Vₘₐₓ/Kₘ, from the intercept on the x-axis.

Statistics

Na/Li CT kinetic parameters are presented as median with the upper and lower quintiles because some of these parameters were not normally distributed. The Mann-Whitney U test was used to assess differences between groups, and the Wilcoxon paired test was used for differences within groups. Clinical values were normally distributed and are presented as mean±SE. Probit values for the frequency distributions were determined from tables. This plot was used descriptively, and the smallest possible number of groups was used to describe the distributions.

Results

Na/Li CT Kinetics in Untreated Erythrocytes

There was a marked kinetic abnormality in Na/Li CT in the group of EHT-FH patients. In untreated erythrocytes, Kₘ of

Methods

Subjects

Fifty-four NCs (24 male and 30 female) were chosen from hospital and university staff and from the local community. None had hypertension (their blood pressures were <140/90 mm Hg), diabetes, or hyperlipidemia, and there was no known family history of these conditions. Thirty-four hypertensive subjects (14 male and 20 female) with normal renal function were chosen from a hospital hypertension clinic (their blood pressures were >160/95 mm Hg, or they were on antihypertensive treatment). They were selected on the basis of a positive family history of hypertension with at least 2 affected first-degree relatives. They also had a family history of early (ie, occurring before the age of 55 years) cardiovascular disease (EHT-FH patients). None of the hypertensive subjects was diabetic or had a family history of diabetes. Thirty of the hypertensive patients were taking regular antihypertensive therapy at the time of the study.

The study was approved by the Joint Ethical Committee of Newcastle Health Authority and University of Newcastle-on-Tyne. All subjects gave informed consent to the study.

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**Na/Li CT Kinetic Parameters and Effect of Thiol Group Alkylation With NEM**

<table>
<thead>
<tr>
<th></th>
<th>NCs</th>
<th>EHT-FH Patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na/Li CT (140 mmol/L sodium)</td>
<td>0.22 (0.16–0.27)</td>
<td>0.27 (0.20–0.32)*</td>
</tr>
<tr>
<td>$V_{max}$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Untreated</td>
<td>0.34 (0.27–0.45)</td>
<td>0.37 (0.31–0.48)</td>
</tr>
<tr>
<td>NEM/choline</td>
<td>0.31 (0.25–0.41)</td>
<td>0.44 (0.34–0.52)†</td>
</tr>
<tr>
<td>$K_m$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Untreated</td>
<td>89 (75–106)</td>
<td>71 (56–94)‡</td>
</tr>
<tr>
<td>NEM/choline</td>
<td>64 (57–77)†</td>
<td>78 (66–89)†</td>
</tr>
<tr>
<td>$V_{max}/K_m$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Untreated</td>
<td>4.0 (3.1–4.8)</td>
<td>5.7 (4.2–6.9)‡</td>
</tr>
<tr>
<td>NEM/choline</td>
<td>5.0 (3.6–6.6)†</td>
<td>6.1 (4.6–7.2)</td>
</tr>
</tbody>
</table>

Values are medians with upper and lower quintiles. Kinetic parameters of $V_{max}$ (mmol Li/h×L rbc), $K_m$ (mmol Na/L), and $V_{max}/K_m$ are for untreated erythrocytes and erythrocytes after thiol group alkylation with NEM in choline as described in Methods. Erythrocytes were from NCs (n=54) or EHT-FH patients (n=34).

*P<0.02 and †P<0.001 compared with NCs; ‡P<0.001 compared with native erythrocytes.

Na/Li CT was lower and $V_{max}/K_m$ was higher in the EHT-FH patients than in NCs (Table, Figure 1). There was no difference in $V_{max}$ of Na/Li CT between these groups of EHT-FH patients and NCs. The single flux rate of Na/Li CT, although slightly higher in EHT-FH patients than NCs, mainly reflected $V_{max}$ and was a poor indicator of the abnormality in $K_m$.

**Effects of NEM on Na/Li CT Kinetics**

There were marked differences between EHT-FH patients and NCs in the effects on Na/Li CT kinetics of a thiol group that reacted rapidly with NEM in the absence of sodium or lithium ions. In NCs, treatment with NEM caused a large decrease in $K_m$ with a small decrease in $V_{max}$, so that $V_{max}/K_m$ was significantly increased (Table). In contrast, in EHT-FH patients, the same NEM treatment paradoxically caused increases in $K_m$ and $V_{max}$, with no change in $V_{max}/K_m$. Thus, after NEM treatment, $K_m$ and $V_{max}/K_m$ values in erythrocytes from NCs approached the values in untreated erythrocytes from EHT-FH patients.

The change in $K_m$ due to NEM treatment of erythrocytes was the most marked abnormality in Na/Li CT in EHT-FH patients compared with NCs (Figure 1).

**Frequency Distributions of Effects of NEM on Na/Li CT Kinetics**

The overlap in values, even for the most discriminating parameter, may have arisen because of heterogeneity within the EHT-FH group; indeed, it is unlikely that selection of patients on simply clinical criteria would give a pure eutiotic subgroup of essential hypertensives. To investigate the possibility that the EHT-FH group might contain discrete subgroups, the frequency distributions of the change in $K_m$ of Na/Li CT after NEM treatment were examined by using probit plots, which display normally distributed values as a single straight line.

These plots showed that the values for change in $K_m$ after NEM in the majority of NCs fell on a single straight line, indicating a homogeneous group with a median decrease in $K_m$ of 24.5 mmol/L. However, in EHT-FH patients, the values did not fit a single distribution. Nine of the patients constituted a group in whom the median decrease in $K_m$ was 24 mmol/L, which was very similar to the value in NCs. The remaining 25 patients (74%) did not fit this group and had changes in $K_m$ with NEM that were rarely seen in NCs (Figure 2).

Probit analysis of the change in $V_{max}$ of Na/Li CT with NEM treatment showed again that the majority of values for NC fell on a single straight line, indicating a homogeneous group with a median decrease in $V_{max}$ of 0.021 mmol Li/h×L rbc. Ten of the EHT-FH patients had values that fitted a similar distribution, with a mean decrease in $V_{max}$ of 0.020 mmol Li/h×L rbc, but the remaining 24 patients (71%) did not fit this distribution and had an abnormal median increase in $V_{max}$ of 0.087 mmol Li/h×L rbc. There was a relation between the abnormal changes in $K_m$ and $V_{max}$ in EHT-FH patients after NEM treatment of erythrocytes (Figure 3) so that mainly the same patients were identified in the abnormal subgroups on both probit plots. Thus, 5 of the EHT-FH patients had a normal response to NEM in both $K_m$ and $V_{max}$.

**Stability of NEM Effect on Kinetic Parameters of Na/Li CT**

In 16 normal subjects measured twice at an interval of 6 months, the values for the change in $K_m$ after NEM treatment are shown as median (quintile range) and were −29 (−36 to −18) and −26 (−30 to −24) mmol Na/L; for the change in $V_{max}$, they were −0.03 (−0.06 to 0.00) and −0.01 (−0.05 to 0.01) mmol Li/h×L rbc. In 5 EHT-FH patients, the NEM-induced changes in $K_m$ were 18 (8 to 21) and 13 (−2 to 15) mmol Na/L, and the change in $V_{max}$ was 0.15 (−0.01 to 0.18) and 0.10 (0.07 to 0.12) mmol Li/h×L rbc.

The corresponding values in untreated erythrocytes were 86 (74 to 97) and 88 (75 to 98) mmol Na/L for $K_m$ and 0.36 (0.26 to 0.40) and 0.31 (0.26 to 0.40) mmol Li/h×L rbc for $V_{max}$ in NCs. In EHT-FH patients, $K_m$ was 62 (49 to 69) and 61 (44 to 62) mmol Na/L, and $V_{max}$ was 0.47 (0.30 to 0.57) and 0.49 (0.24 to 0.49) mmol Li/h×L rbc.

**Relation of Na/Li CT Parameters to Clinical Values**

Serum triglycerides were higher in EHT-FH patients (1.65±0.15 mmol/L) than in NCs (1.07±0.15 mmol/L), but there was no difference in serum cholesterol (5.19±0.23 versus 5.24±0.25 mmol/L), and body mass index was similar (26.8±1.10 versus 23.8±0.68 kg/m²). In EHT-FH patients, there was a correlation between plasma triglycerides and both $V_{max}$ ($r=0.37, P<0.01$) and the single Na/Li CT flux rate at 140 mmol/L sodium ($r=0.32, P<0.02$). Thus, high single Na/Li CT flux rate values were mainly related to high plasma triglycerides. In contrast, $K_m$ and the change in $K_m$ with NEM treatment was not related to plasma triglycerides, so that these parameters were not confounded by this metabolic component. No effect of gender on Na/Li CT kinetic parameters was detected in these groups of subjects.
The EHT-FH group had higher blood pressure, and most patients were taking regular antihypertensive therapy. There is no evidence that the level of blood pressure or that antihypertensive medications affect Na/Li CT activity, although abnormal Na/Li CT may be a marker for a more severe type of hypertensive disease. The low Km abnormality of Na/Li CT is present in first-degree relatives of patients with either EHT-FH or diabetic nephropathy. In addition, half of a group of type 2 diabetic patients receiving antihypertensive treatment had completely normal Na/Li CT kinetic parameters. The data for the subgroup of EHT-FH patients who were receiving antihypertensive medication and had normal Na/Li CT parameters in the present study agree with these conclusions.

**Discussion**

The sensitivity to thiol-alkylating agents is the only information available concerning a protein moiety linked to Na/Li CT activity.
CT. One of the thiol groups alkylated has a very low pK and reacts with NEM in the absence of sodium or lithium ions to alter the kinetic parameters of Na/Li CT in normal erythrocytes, as expected if this thiol protein is responsible for the abnormal Na/Li CT in EHT-FH patients. Thus, in erythrocytes from NCs, NEM causes a decrease in $K_m$ and an increase in $V_{max}/K_m$, but in erythrocytes from EHT-FH patients, in which $K_m$ is already low and $V_{max}/K_m$ is high, NEM does not cause a further change in these parameters. This is in contrast to the thiol alkylation with NEM in the presence of sodium or lithium ions, which causes a large decrease in $V_{max}/K_m$, and cannot explain the raised $V_{max}/K_m$ in EHT-FH patients. This suggests that the thiol group that reacts rapidly with NEM in the absence of sodium or lithium is responsible for the abnormal Na/Li CT kinetics in EHT-FH patients.

Similar to most previous studies, there is much overlap in Na/Li CT parameters (Figure 1), but the effect of thiol alkylation with NEM on $K_m$ was the clearest discriminator in the EHT-FH patients. It was unaffected by plasma triglyceride levels. This contrasts with the single Na/Li CT flux rate at 140 mmol/L sodium, which was related to plasma triglyceride levels as a result of the effect of plasma triglycerides on $V_{max}$. Thus, in studies of Na/Li CT in hypertension, the single flux rate measurement at 140 mmol/L sodium will be confounded by variations in plasma triglycerides. It is clear that in the present study the EHT-FH patients with the abnormal Na/Li CT kinetic parameters that were due to the abnormal thiol protein were very poorly discriminated by the single Na/Li CT flux rate assay. A recent meta-analysis of data from the latter assay concluded that the difference assessed by Na/Li CT flux rate between patients with EHT and NCs was weak but observed that kinetic parameters may be more useful, and this is clearly shown in the present study.

The frequency distributions of the effect of NEM on $K_m$ of Na/Li CT were plotted by using the probit values to seek evidence of homogeneity within the groups. A single normal distribution will be shown as a single straight line in the probit plot. This showed that the values for the NCs were close to a single normal distribution, whereas the EHT-FH patients were not homogeneous for this parameter. Therefore, even in this subgroup of hypertensive patients selected on strict clinical criteria, the abnormality in Na/Li CT was not homogeneous. In a minority of the patients, the response of the Na/Li CT kinetic parameters to NEM was completely normal, and they were indistinguishable from the rest of the patients for the clinical measurements that were made. The proportion of patients in each of the 2 subgroups is descriptive only of the subjects in the present study and is likely to vary between different groups selected on clinical criteria; this situation is likely to explain some of the variability between studies when overall mean values are presented.

However, the similar medians for the change in $K_m$ of Na/Li CT in NCs and one subgroup of EHT-FH patients may indicate wider validity for these values. Repeated measurements of kinetic parameters in NCs and patients indicated that the effect of NEM was a stable characteristic.

In contrast to NCs, in whom NEM caused a decrease in $K_m$ with an increase in $V_{max}/K_m$, in the EHT-FH patients the paradoxical increase in $K_m$ caused by NEM was accompanied by an increase in $V_{max}$, so that $V_{max}/K_m$ did not change. Our interpretation of these changes is that in NCs, alkylation of the key thiol protein increases the sodium ion binding rate constant (decreased $K_m$ and increased $V_{max}/K_m$), whereas in EHT-FH patients, it increases the turnover rate of the transporter (increased $V_{max}$ and $K_m$ and constant $V_{max}/K_m$). The unchanged $V_{max}/K_m$ shows that a change in sodium ion binding rate is very unlikely. Thus, the key thiol protein transporter is organized differently in EHT-FH patients compared with NCs.

This difference in transporter organization is deduced from the kinetics of Na/Li CT, in which the rate constant for ion translocation is significant relative to that for ion binding, especially of sodium. Therefore, the Michaelis constant ($K_m$) for external sodium not only reflects sodium affinity at this site but also varies directly with the turnover rate of the transporter. If the number of transporter units is constant, which may be assumed over a short period in vitro, the $V_{max}/K_m$ ratio is a measure of the ion association rate constant.
and gives the best assessment of ion binding events. If the rate of turnover changes, then \( V_{\text{max}} \) will change, and \( K_{m} \) will also change in proportion.

Abnormal Na/Li CT is associated with more severe hypertension and metabolic complications such as insulin resistance. Clearly, all of these factors will cosegregate with abnormal Na/Li CT, but that does not allow causality to be inferred. Indeed, there is a strong inherited component to the activity of Na/Li CT,\(^{23} \) and it is strongly linked to the inheritance of hypertension.\(^{24,25} \) with abnormal Na/Li CT activity found in normotensive first-degree relatives.\(^{18,25} \) However, its molecular nature is unknown, and without a candidate protein, molecular genetic studies are not possible. A genetic study founded on the hypothesis that the Na\(^+\)-H\(^+\) exchanger NHE1 was mediating Na/Li CT excluded NHE1 from linkage with EHT with very high confidence.\(^{26} \) However, because rat erythrocytes have very high NHE1 activity but no Na/Li CT activity,\(^{3} \) this hypothesis was unlikely to be true. No other candidate proteins have been suggested in relation to Na/Li CT.

We conclude that an abnormal thiol protein could be responsible for the abnormal Na/Li CT kinetics in a subgroup of EHT patients who may have a common biochemical pathology. The identification of this thiol protein will provide the first protein directly linked to Na/Li CT. This may facilitate genetic studies and elucidation of the pathogenesis of EHT in this group of patients. A single flux rate assay for Na/Li CT will not identify these patients; the measurement of kinetic parameters is required.

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