Mixture Analysis of Erythrocyte Lithium-Sodium Countertransport and Blood Pressure

Alan B. Weder and Nicholas J. Schork

This study employs multivariate normal mixture analysis, a technique for identifying discrete subgroups within populations, to examine the relation of erythrocyte lithium-sodium (RBC Li\(^+\)-Na\(^+\)) countertransport and blood pressure in a group of 474 healthy adults. After adjusting for effects of age, gender, race, height, and weight, univariate mixture analysis of the distribution of mean arterial blood pressure (MAP) revealed the presence of only one group, whereas the distribution of RBC Li\(^+\)-Na\(^+\) countertransport values was composed of a mixture of two groups (p<0.00005). When bivariate mixture analysis was applied to the combined distribution of MAP and RBC Li\(^+\)-Na\(^+\) countertransport, two commingled subgroups were identified (p<0.00005). The smaller group (19%) had significantly higher values for both MAP (108.7±16.7 mm Hg, mean±SD) and RBC Li\(^+\)-Na\(^+\) countertransport (0.455±0.147 mmol Li\(^+\)/1 cells • hr) than the larger (81%) group (MAP 93.3±12.2 mm Hg, RBC Li\(^+\)-Na\(^+\) countertransport 0.247±0.080 mmol Li\(^+\)/1 cells • hr, p<0.0001 for both differences). The relation of MAP to RBC Li\(^+\)-Na\(^+\) countertransport was distinctly different in these two subgroups. In the larger group, we found a weak positive (r=0.21, p<0.0001) correlation for unadjusted values, which was not significant after adjustment. The smaller group, with higher levels of MAP and RBC Li\(^+\)-Na\(^+\) countertransport, showed significant negative correlations for both unadjusted (r= -0.28, p<0.008) and adjusted (r=−0.41, p<0.0001) values. (Hypertension 1989;13:145-150)

Essential hypertension is clearly heritable, but its specific biochemical lesions are obscure and their genetic determinants unknown. Studies in twins and in relatives of hypertensive individuals have proven that several physiological processes relating to blood pressure control, which include sympathetic nervous system function, plasma renin activity, excretion of a sodium load, and the degree of sodium sensitivity of blood pressure, are heritable, but these phenomena are too complex to have yet yielded to a more detailed mechanistic analysis at cellular or molecular levels. Other relatively simple heritable biochemical markers associated with hypertension (MN blood group haplotypes or sodium sensitivity (haptoglobin phenotypes) have no obvious physiological relation to blood pressure control. Of greater promise is elevated red blood cell lithium-sodium (RBC Li\(^+\)-Na\(^+\)) countertransport, a recently proposed marker for essential hypertension that has been shown to be associated with high blood pressure in many studies and also to be related to organ-scale abnormalities of potential pathogenetic importance to hypertension. Recently, Turner et al demonstrated that the distribution of values of RBC Li\(^+\)-Na\(^+\) countertransport in a normotensive group of blood bank donors could be resolved into two normal subdistributions. It was suggested that individuals in the higher Li\(^+\)-Na\(^+\) countertransport subdistribution could be at increased genetic risk for future hypertension, although an association between elevated countertransport and high blood pressure could not be proven because individuals with established hypertension were excluded. We examined the relation of RBC Li\(^+\)-Na\(^+\) countertransport and blood pressure in a combined population of normotensive and hypertensive subjects by using a novel bivariate analytic technique to test the hypothesis that high blood pressure cosegregates with elevated RBC Li\(^+\)-Na\(^+\) countertransport.

Subjects and Methods

The population studied consisted of 474 healthy adults who had participated in a variety of studies at the University of Michigan Hypertension Division.
from 1983–1987. These subjects were not randomly selected, but included all individuals for whom standardized blood pressure and RBC Li\(^+\)-Na\(^+\) countertransport measures were available. Generally, participants had been recruited by blood pressure level, and most studies compared untreated, borderline (at least one casual blood pressure measurement >140/90 mm Hg and at least one <140/90 mm Hg within the preceding year) or mild (average casual blood pressure >140/90 mm Hg) hypertensive subjects with normotensive control subjects (blood pressure <140/90 mm Hg). No subjects with recognized coronary or cerebrovascular disease, renal insufficiency, or endocrine or liver disease participated in any study, and all had abstained from antihypertensive drugs and estrogen-containing preparations for at least 4 weeks. For all subjects, blood pressure measurement was performed in the seated position after at least 10 minutes quiet rest. Blood pressure was determined three times with a mercury sphygmomanometer and a cuff of appropriate size for the individual’s arm; the average was used to calculate mean arterial blood pressure (MAP) as diastolic blood pressure plus one third of the pulse pressure. In addition to MAP, the height, weight, gender, age, and race (self-determined) were recorded. Blood for RBC Li\(^+\)-Na\(^+\) countertransport, obtained on the same day as blood pressure was measured, was assayed by the method of Canessa et al\(^,7\) as previously reported for our laboratory.\(^8\) No preselection based on RBC Li\(^+\)-Na\(^+\) countertransport activity was exercised.

**Statistical Methods**

Values are reported as mean±SD. The significance of group differences was assessed with Student’s \(t\) test and by \(\chi^2\) when appropriate. Significance was accepted at \(p\leq0.05\). The technique of mixture analysis has been described rigorously,\(^21,22\) and the present application is similar to previously worked examples (A.B. Weder, N.J. Schork, and M.A. Schork, unpublished observations). Briefly, data were first adjusted to eliminate the effects of measured concomitant variation. Race and gender, along with the first-, second-, and third-order (i.e., power) effects of weight, height, and age, and all combinations of these variables (to allow for possible interaction effects) were entered into a stepwise regression algorithm as independent variables with blood pressure and RBC Li\(^+\)-Na\(^+\) countertransport as separate dependent variables. Those variables that contributed significantly (\(p<0.05\) by \(F\) test) to overall variability in blood pressure and countertransport activity were retained in a final multiple regression model, which was performed with forward selection, that is, the variable explaining the most variability was entered first, that explaining the most residual variability next, and so on until all significant variables were entered. The residual values from these empiric models, which represented variability not explained by demographic or body size factors, were generated for subsequent mixture analyses after adding back the mean values of the unadjusted variables to aid in interpretation. Next, the individual distributions of these derived values for MAP and RBC Li\(^+\)-Na\(^+\) countertransport were examined for the presence of mixtures with simultaneous adjustment for skewness. Adjustment for skewness is a critical component of mixture analysis because skewness alone can result in the appearance of a mixture.\(^22\) The optimal number of mixed distributions within each population was determined by \(\chi^2\) testing. Finally, bivariate analysis was applied to the combined distribution of MAP and RBC Li\(^+\)-Na\(^+\) countertransport, again with simultaneous adjustment for skewness and likelihood ratio testing.

Standard deviations of the proportions classified into each group and the group parameter means and standard deviations were derived from an analysis of 200 bootstrap samples.\(^23\) These bootstrap errors generally represent the “stability” of the mixture (i.e., that the mixture is not due to outliers or statistical “noise”) with small errors indicating stability.\(^24\)

**Results**

The characteristics of the study population are shown in Table 1. The data adjustments undertaken to account for the possible confounding effects of measured demographic and body size factors explained a total of 39.6% of the initial variability in MAP and 15.1% of the variability in RBC Li\(^+\)-Na\(^+\) countertransport rates. The contributions that were significant for individual variables, power effects of individual variables, and interactions between variables are shown in Table 2.

Figures 1 and 2 show the frequency distributions for unadjusted and adjusted values for MAP and RBC Li\(^+\)-Na\(^+\) countertransport. Mixture analysis of the adjusted MAP distribution demonstrated the presence of a single population (Table 3). In agreement with the report of Turner et al\(^,26\) the distribution of adjusted Li\(^+\)-Na\(^+\) countertransport values showed a mixture of two subgroups (Table 3). The presence of two groups did not result from skew-
ness in the original distribution, as two subpopulations were still present when skewness was controlled during the mixture analysis.

Bivariate mixture analysis of MAP and RBC Li⁺-Na⁺ countertransport demonstrated a highly significant ($p<0.00005$) probability that two subpopulations were commingled in our sample (Table 3). The smaller subpopulation (19±13%) had higher levels of MAP and RBC Li⁺-Na⁺ countertransport than the larger (81±13%). Table 4 shows the characteristics of the two groups as defined by this bivariate commingling analysis.

We have previously reported\textsuperscript{16,19} a positive correlation between unadjusted, seated MAP and RBC Li⁺-Na⁺ countertransport, and a similar relation was present in the entire population for both the unadjusted ($r=0.27, p<0.0001$) and the adjusted ($r=0.16, p<0.0003$) values of those variables. However, when the two subgroups identified by mixture analysis were examined separately, qualitatively different relations were found. In the larger group, characterized by lower MAP and countertransport values, a significant positive relation was found for unadjusted values ($r=0.21, p<0.0001$) but was reduced to nonsignificance ($r=0.06, p=0.19$) by adjustment for measured concomitants. However, in the smaller group with higher MAP and countertransport levels, a significant inverse relation was found for both unadjusted ($r=-0.28, p<0.008$) and, as shown in Figure 3, adjusted ($r=-0.41, p=0.0001$) values. We hypothesize that the positive correlation found in the original, entire population may be a manifestation of the "polarization" of the two groups into distinct corners of the MAP/RBC Li⁺-Na⁺ countertransport space.

**Discussion**

The present study demonstrates a cosegregation of high values of RBC Li⁺-Na⁺ countertransport and blood pressure in our study group. Although we studied a population of individuals who were initially selected for blood pressure levels, univariate examination of blood pressure values in the entire sample revealed the presence of a single group. It is therefore highly unlikely that the subsequent finding of a mixture in the bivariate analysis results from subject preselection. Univariate analysis of RBC Li⁺-Na⁺ countertransport did reveal two groups, in agreement with an earlier report that examined a randomly selected group of normotensive blood bank donors.\textsuperscript{20} In that study, a trend toward an association of high Li⁺-Na⁺ countertransport activity and high blood pressure was noted, and our observations confirm that high RBC Li⁺-Na⁺ countertransport is a marker for hypertension.

**TABLE 2. Stepwise Multiple Regression Model Showing Contribution of Independent Variables to Variability in Mean Arterial Blood Pressure and Erythrocyte Lithium-Sodium Countertransport**

<table>
<thead>
<tr>
<th>Independent variable</th>
<th>MAP Cumulative $r^2$</th>
<th>p</th>
<th>MAP Cumulative $r^2$</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight</td>
<td>0.225</td>
<td>&lt;0.00001</td>
<td>Gender</td>
<td>0.077</td>
</tr>
<tr>
<td>Age</td>
<td>0.320</td>
<td>&lt;0.00001</td>
<td>Race</td>
<td>0.102</td>
</tr>
<tr>
<td>Gender</td>
<td>0.339</td>
<td>0.0002</td>
<td>Weight</td>
<td>0.118</td>
</tr>
<tr>
<td>Height</td>
<td>0.355</td>
<td>0.0005</td>
<td>Weight\textsuperscript{3}</td>
<td>0.132</td>
</tr>
<tr>
<td>Height × Age</td>
<td>0.369</td>
<td>0.0018</td>
<td>Race × Weight</td>
<td>0.144</td>
</tr>
<tr>
<td>Gender × Age</td>
<td>0.375</td>
<td>0.0301</td>
<td>Height</td>
<td>0.151</td>
</tr>
<tr>
<td>Height × Weight × Age</td>
<td>0.380</td>
<td>0.0436</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weight × Age\textsuperscript{2}</td>
<td>0.387</td>
<td>0.0279</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Height × Age\textsuperscript{2}</td>
<td>0.396</td>
<td>0.0084</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Independent variables are measured demographic and body size factors. Mean arterial pressure (MAP) and lithium-sodium (Li⁺-Na⁺) countertransport are dependent variables.

**FIGURE 1. Bar graphs showing the distribution of unadjusted (panel A) and adjusted (panel B) mean arterial blood pressure in the study group. As indicated by the overlay, a single normal distribution provides the best fit for the data.**
The importance of this finding is twofold. First, normal mixture analysis allows us to define, by means of a single biochemical marker, a distinct subgroup of hypertensive individuals in whom hypotheses of possible biochemical or physiological mechanisms underlying hypertension can be tested. Second, although the presence of a mixture alone can never prove that a genetic process is operative, in light of the strong genetic determination of RBC Li\(^+-\)Na\(^+\) countertransport variability, the presence of a mixture in the distribution of this trait does suggest the possibility that a single gene or group of genes controls the phenotypic expression of countertransport activity. The finding that one subgroup has both high Li\(^+-\)Na\(^+\) countertransport and hypertension strongly supports the potential use of countertransport as a genetic marker of hypertension. Although the molecule effecting transmembrane Li\(^+-\)Na\(^+\) countertransport in RBCs has not been isolated, it seems likely to be a single intrinsic membrane protein coded by a single gene. The clear association of this gene product with hypertension, if confirmed by pedigree studies, should provide a reliable genetic marker for studies of the heritable basis of human hypertension, whether it is expressed in cardiovascular effector tissues where it could directly cause hypertension, or not.

Although mixture analysis unequivocally demonstrates that there are two commingled subgroups in the population studied, it should be pointed out that the proportions classified into, and the particular demographic and body size characteristics attributed to, each group may vary with subject selection. To clearly define the prevalence of individuals in each subpopulation, a large, population-based survey of RBC Li\(^+-\)Na\(^+\) countertransport and blood pressure will be required. However, the mean RBC Li\(^+-\)Na\(^+\) countertransport values in the two groups we identified (0.247 and 0.455 mmol Li\(^+\)/1 cells \(\cdot\) hr) are very similar to the values reported by Turner et al\(^{20}\) (0.24 and 0.42 mmol Li\(^+\)/1 cells \(\cdot\) hr) for subgroups detected in a population of normotensive blood bank donors. The agreement in the two studies, and the further agreement of both sets of values with the average countertransport values reported in many case-control comparisons of normotensive and hypertensive subjects (see Reference 20) suggests that elevated Li\(^+-\)Na\(^+\) countertransport is a phenotype with limited interpopulation variability and reliable association with hypertension.

It has been noted that the use of an efflux medium in which Mg\(^{2+}\) is substituted for Na\(^+\) can result in partial suppression of Li\(^+\) efflux.\(^{26}\) The consequent overestimation of Li\(^+-\)Na\(^+\) countertransport, which is calculated as the difference in Li\(^+\) efflux in Na\(^+\) and Mg\(^{2+}\) media, may account for the observed differences in countertransport values reported in different studies. However, the agreement of our results with those of Turner et al\(^{20}\) suggests that the efflux medium used in our study was adequate for the purposes of this investigation.

### Table 3. Univariate and Bivariate Mixture Analyses for Mean Arterial Blood Pressure and Erythrocyte Lithium-Sodium Countertransport

<table>
<thead>
<tr>
<th>Analytic approach, variables (adjusted values)</th>
<th>Group 1</th>
<th>Group 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>% of total</td>
<td>Mean</td>
<td>SD</td>
</tr>
<tr>
<td><strong>Univariate</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MAP</td>
<td>100</td>
<td>96.1</td>
</tr>
<tr>
<td>RBC Li(^+-)Na(^+) countertransport</td>
<td>73%±16</td>
<td>0.009±0.137</td>
</tr>
<tr>
<td><strong>Bivariate</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MAP</td>
<td>81%±13</td>
<td>95.9±0.1</td>
</tr>
<tr>
<td>RBC Li(^+-)Na(^+) countertransport</td>
<td>81%±13</td>
<td>0.004±0.122</td>
</tr>
</tbody>
</table>

Standard deviation of the proportions and group means and standard deviations were derived from 200 bootstrap samples.\(^{21,24}\) MAP, mean arterial blood pressure; NS, not significant; RBC Li\(^+-\)Na\(^+\), erythrocyte lithium-sodium. *Significance of likelihood (\(\chi^2\) with 4 degrees of freedom) of two (vs. one) groups, with adjustment for skewness.
TABLE 4. Characteristics of Two Groups Defined by Bivariate Mixture Analysis

<table>
<thead>
<tr>
<th>Variable (unadjusted values)</th>
<th>Group 1</th>
<th>Group 2</th>
<th>p*</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>384</td>
<td>90</td>
<td></td>
</tr>
<tr>
<td>Age (yr)</td>
<td>33.7±7.6</td>
<td>35.2±9.6</td>
<td>NS</td>
</tr>
<tr>
<td>Gender (M:F)</td>
<td>263:121</td>
<td>75:15</td>
<td>0.005†</td>
</tr>
<tr>
<td>Race (W:B)</td>
<td>343:82</td>
<td>41:8</td>
<td>NS†</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>78.8±16.7</td>
<td>85.5±17.9</td>
<td>0.001</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>173.9±9.3</td>
<td>176.3±8.6</td>
<td>0.03</td>
</tr>
<tr>
<td>Seated blood pressure (mm Hg)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Systolic</td>
<td>123.0±15.8</td>
<td>140.9±20.7</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Diastolic</td>
<td>78.4±12.0</td>
<td>92.6±15.8</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Mean</td>
<td>93.3±12.2</td>
<td>108.7±16.7</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>RBC Li⁺⁻Na⁺ countertransport (mmol Li⁻/1 cells • h)</td>
<td>0.247±0.080</td>
<td>0.455±0.147</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

n, number of subjects; M, male; F, female; W, white; B, black; RBC Li⁺⁻Na⁺, red blood cell lithium-sodium.

*Significant by Student’s t test, except as noted.
†Significant by χ².

Mg²⁺ media, could contribute to skewing of the Li⁺⁻Na⁺ distribution toward higher values, particularly if the suppressive effect of Mg²⁺ is most prominent in subjects with the highest countertransport values. However, as we controlled for skewness in our analysis, our finding of a mixture was probably not affected by this problem in methodology.

We and others have previously reported a positive correlation between blood pressure and RBC Li⁺⁻Na⁺ countertransport. Subgroup analysis in the present study suggests that this correlation results from the presence of two subgroups, one with relatively low values for blood pressure and countertransport and the other with higher values for both measures. When the subgroups are analyzed separately, the residual weak correlation found in the low-MAP, low-countertransport group is abolished by adjustment for demographic and body size measures. For the smaller group with higher MAP and countertransport, however, the relation is inverse and is actually improved by adjustment. We can offer no explanation for the cause of this correlation, as the mechanisms relating high RBC Li⁺⁻Na⁺ countertransport and hypertension are still uncertain, but the finding of a significant inverse relation in the subgroup with higher MAP provides further support for the contention that Li⁺⁻Na⁺ countertransport or an analog system is intimately linked to the pathological basis of hypertension in some individuals. In this regard, the recent observations reported by Canessa et al. that RBC Li⁺⁻Na⁺ countertransport may be a mode of Na⁺⁻H⁺ exchange and by Livne et al. that platelet Na⁺⁻H⁺ exchange is increased in essential hypertension suggest that abnormalities of the ubiquitous transmembrane Na⁺⁻H⁺ exchange may be a fruitful area for future research into the cellular mechanism causing human hypertension.

In summary, we have demonstrated that high RBC Li⁺⁻Na⁺ countertransport activity cosegregates with high blood pressure independent of the effects of age, race, gender, body weight, or height. Furthermore, there is a highly significant inverse correlation between countertransport and blood pressure in the group with high values of both variables that is not seen in the group with lower countertransport and blood pressure levels. These findings support the hypothesis that there is a distinct subgroup of hypertensive individuals in whom elevated RBC Li⁺⁻Na⁺ countertransport is a marker of a genetic predisposition to hypertension.

References


KEY WORDS • lithium-sodium countertransport • erythrocytes • essential hypertension
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