Red Cell Cotransport Activity and Sodium Content in Black Men
Relationship to Essential Hypertension

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SUMMARY Furosemide-sensitive sodium and potassium cotransport and intracellular sodium content ([Na+]i) were measured in erythrocytes (red blood cells, RBCs) from a population of 90 adult black men with and without essential hypertension (EH). The mean values for sodium cotransport activity, expressed as furosemide-sensitive Na efflux (mmol/liter RBC/hr), were not significantly different among the EH patients and two control groups, normotensive subjects with a positive history (N+) and those with a negative family history (N-) for hypertensive disease (EH: 154 ± 123, n = 53; N+:167 ± 93, n = 12; and N−: 207 ± 142, n = 20; all values are means ± SD). The mean [Na+]i, 9.66 ± 3.02 mmol/liter RBC (n = 56) for the EH group was greater than the mean value for the N− control group (7.96 ± 1.97, n = 20; p < 0.05). The N+ group also displayed a higher mean [Na+]i, (10.38 ± 3.18, n = 12; N+ vs N− p< 0.01). Although there was substantial overlapping of [Na+]i values between the groups and no clear dividing line, the distribution curve of the [Na+]i values in EH was skewed toward higher concentrations than in N−. Nevertheless, we must conclude that erythrocyte cotransport and [Na+]i are not clinically useful in the identification of EH in black men.

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KEY WORDS • essential hypertension • sodium • erythrocyte

ESSENTIAL hypertension (EH) is a major public health problem in industrialized countries. It is generally accepted that heredity plays an important role in the etiology of EH although the extent of involvement is still unresolved.1 Studies2 in strains of rats that become hypertensive on high salt diets (Dahl salt-sensitive rats) have demonstrated a genetically transmitted defect of renal sodium (Na) excretion, the hypertensive effects of which are humorally mediated. The cellular factors responsible for the Na excretion deficiency have not been identified, but the involvement of renal cellular cation transport has been suspected. The idea that this inherited abnormality of cellular Na metabolism also may be present in more accessible cells has lead to the search for markers of EH in blood cells.

A variety of abnormalities of red blood cell (RBC) cation metabolism have been identified in individuals with EH and their offspring.3-5 The activity of the furosemide-sensitive, Na + K cotransport system has been proposed1 as a marker for EH. These studies have demonstrated a reduction of RBC furosemide-sensitive Na efflux in EH and have suggested a genetic origin by finding this defect in the normotensive offspring of EH patients. In support of a primary genetic, rather than a physiological, basis for this transport deficiency is the demonstration of normal cotransport in persons with secondary hypertension.3-4 It has been suggested that RBC cotransport activity measurement might therefore be used as a screening tool to identify persons at risk for developing EH.

Some investigators6,7 have been unable to detect cotransport abnormalities in their subjects with EH. Others8 have even demonstrated an elevated RBC cotransport in patients with EH compared to normotensive controls. Many of these studies have been conducted on heterogenous populations of unspecified racial origin and have included males, females, adolescents, and adults. Furthermore, technique differences make certain comparisons problematic.
The prevalence of EH is exceptionally high in American black men, particularly in the inner city, where the frequency is almost 25%. The RBC Na metabolism has not been studied in this population. Therefore, we have used the pCMBS (parachloromercuribenzenesulfonate) loading technique of Dagher and Garay to study the RBC cotransport activity of a large population of adult black men. We also measured the RBC Na content, because previous studies in white populations have shown elevated levels among patients with EH.

Methods

Patients

Individuals with EH were all previously diagnosed and followed at the Hypertension Clinic of the Loch Raven Veterans Administration Medical Center, Baltimore, Maryland. All were black men over 30 years of age with no other systemic illnesses. Criteria for diagnosis and inclusion in the study required a systolic blood pressure (BP) greater than 145 mm Hg and a diastolic BP greater than 90 mm Hg on three or more clinical visits. Chest radiograph, serum electrolytes, blood urea nitrogen, creatinine, and urinalysis were normal. All of these patients previously had been controlled on antihypertensive medication and were taken off therapy for more than 2 weeks prior to blood collection and were hypertensive at that time. Informed consent was obtained in keeping with the guidelines of the University of Maryland School of Medicine committee on human investigation.

Blood samples were collected in the morning between 9:30 and 11:00 a.m. No special instructions or diets were given. Blood pressures were measured in the sitting position prior to blood sampling. Patients were interviewed regarding the incidence of hypertension or stroke in their first-degree relatives.

Control Subjects

The control population consisted of black male volunteers from the University of Maryland Hospital and Veterans Administration Hospital communities. All were older than 30 years of age and without systemic illnesses or current medication intake. Criteria for inclusion were a systolic BP less than 140 mm Hg and a diastolic BP less than 90 mm Hg and a knowledge of the medical histories of all first-degree relatives (siblings, children, and parents). Normotensive individuals with a negative family history for hypertension or stroke comprised the N− group and those with a positive family history, the N+ group.

Blood samples were collected in the morning between 8:30 and 11:00 a.m. No special instructions or diets were requested. The BPs in the sitting position were obtained prior to blood collection.

Laboratory Techniques

Blood (30 ml) was withdrawn directly into heparinized glass tubes (Venohlet No. T-200SLH) and refrigerated until cell preparation was begun 4 hours later. The technician performing the assays was unaware of the subject's clinical status. All solutions were prepared and assays performed in plastic laboratory ware to prevent leaching of sodium from glassware. Reagents were ACS grade or better. Cysteine, ouabain, adenine, inosine, Hepes, Tris, and EGTA were purchased from Sigma Chemical Company (St. Louis, Missouri). Furosemide was generously supplied by Hoechst-Roussel Pharmaceuticals, Inc. (Somerville, New Jersey).

Measurement of Red Cell Sodium Content

The RBCs were separated from heparinized blood samples by centrifugation at 1700 g for 5 minutes. The plasma was removed, and the buffy coat was discarded. The RBCs were then rinsed three times with iced MgCl2 wash solution (105 mM MgCl2, 10 mM Tris base adjusted to pH 7.8 with HCl, and then to pH 7.4 with Hepes, 290 mOsm) after which the cells were resuspended in the MgCl2 wash solution and a hemato-crit was obtained. The exact volume of cell suspension that yielded 0.125 ml of RBCs was then pipetted into plastic tubes in triplicate. The suspension was centrifuged, the supernatant removed, and the cells lysed with deionized glass distilled water. Without removal of the membranes, the resulting solution was diluted, and the Na and K concentrations were measured by atomic absorption spectrophotometry (Instrumentation Laboratories, AAS No. 457). We included 50 mM CsCl in samples for Na determinations to suppress ionization. We then calculated the intracellular concentrations in millimoles per liter of RBCs (mmol/liter RBC). We made no correction for extracellular-trapped volume.

Determination of Sodium and Potassium Cotransport

Furosemide-sensitive Na and K fluxes mediated by the cotransport system were determined by using a slight modification of the methods described by Dagher and Garay. Approximately 10 ml of heparinized whole blood was centrifuged at 1700 g for 5 minutes, and the plasma and buffy coat were removed. The RBCs were washed twice with 0.9% NaCl, and 3.5 ml of the packed erythrocytes were suspended in 20 ml of the loading medium formulated by Dagher and Garay (60 mM NaCl, 3 mM KCl, 150 mM choline chloride, 1.3 mM N^ + HPQ, 1.3 mM NaH2PO4, 1 mM MgCl2, 1 mM EGTA, and 0.02 mM pCMBS, 390 mOsm, pH 7.4).
3.5), and stored at 4°C for 17 hours without agitation. The suspension was then centrifuged at 1700 g for 6 minutes, and the supernatant was discarded. The treated RBCs were resuspended in 20 ml of cysteine recovery medium (150 mM NaCl, 1 mM MgCl₂, 2.7 mM Na₂HPO₄, 2.7 mM NaH₂PO₄, 10 mM glucose, 1 mM EGTA, 2 mM adenine, 3 mM inosine, and 4 mM cysteine adjusted to pH 7.4 with Tris buffer, 310 mOsM) and incubated at 37°C for 1 hour. The recovery medium was removed, and the RBCs were washed six times with the cold MgCl₂ wash solution (see above). Then 2 ml of incubation medium (78.5 mM MgCl₂, 85 mM sucrose; 5 mM glucose; 0.1 mM ouabain, and 10 mM Tris buffer, adjusted to pH 7.2 with HCl and Hepes, 320 mOsM) was added to the approximately 2 ml of RBCs remaining after six rinses. A hematocrit of this suspension was then obtained to calculate RBC volume. Two 100 μl aliquots were also removed and lysed in 4.0 ml of distilled water to measure intracellular cation concentrations following the loading and recovery periods. Then 1.6 ml of the above suspension was added to 12.0 ml of the incubation medium in the presence or absence of 1 mM furosemide (pH 7.2). These suspensions were each added into three tubes of 3.75 ml and incubated at 37° C. Samples were removed from the water bath at 30, 60, and 90 minutes, cooled on ice, and centrifuged at 1700 g for 6 minutes at 4° C. The supernatants were saved for Na and K concentration determination by atomic absorption spectrophotometry.

Calculations and Statistics

We calculated Na and K efflux rates from the slopes by linear regression (Figure 1). The coefficient of correlation (r) for all assays was greater than 0.992. Passive permeability was calculated as a rate coefficient (k), namely, ouabain-resistant and furosemide-resistant Na or K efflux (μmol/liter RBC/hr) divided by the initial (after-loading) intracellular cation concentration. The reproducibility of each assay for a given subject over time was also evaluated. Eight individuals, had multiple (mean = 6.4) analyses over 1 to 7 months (mean = 4.3 months). None of these subjects were on hypertensive medications, and individuals from each category were included. These data were then used to estimate the assay's variability for an individual. The data are expressed as the mean value of the particular assay for the eight subjects ± the averaged standard deviations (Table 2).

All data are presented as means ± 1 standard deviation (SD). Comparisons of [Na], values were analyzed by using a log transformation in order to make the variances more nearly equal and to bring the skewed distributions toward normality. Statistical significance among the three groups (EH, N⁺, and N⁻) was calculated by using three separate conventional Student’s t-tests for comparison of means. The Bonferroni t statistic was employed to correct p values for the multiple inferences being made. Significance was considered at p < 0.05.

### TABLE 2. Results of Analysis of Variance

<table>
<thead>
<tr>
<th>Subject No.</th>
<th>No. of studies</th>
<th>MAP (mm Hg)</th>
<th>[Na] (mmol/liter RBC)</th>
<th>Na Cotr (p.mol/liter RBC/hr)</th>
<th>K Cotr (μmol/liter RBC/hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>8</td>
<td>99±7</td>
<td>5.85±0.86</td>
<td>355±128</td>
<td>318±94</td>
</tr>
<tr>
<td>2</td>
<td>7</td>
<td>103±3</td>
<td>9.01±0.86</td>
<td>107±48</td>
<td>145±54</td>
</tr>
<tr>
<td>3</td>
<td>5</td>
<td>105±6.3</td>
<td>16.7±1.97</td>
<td>148±27</td>
<td>250±47</td>
</tr>
<tr>
<td>4</td>
<td>6</td>
<td>105±4.9</td>
<td>10.32±1.05</td>
<td>169±65</td>
<td>209±96</td>
</tr>
<tr>
<td>5</td>
<td>7</td>
<td>103±5.1</td>
<td>8.99±0.43</td>
<td>233±79</td>
<td>353±69</td>
</tr>
<tr>
<td>6</td>
<td>5</td>
<td>117±7.2</td>
<td>7.52±0.22</td>
<td>176±62</td>
<td>254±55</td>
</tr>
<tr>
<td>7</td>
<td>7</td>
<td>99±8.8</td>
<td>16.3±1.17</td>
<td>151±71</td>
<td>318±66</td>
</tr>
<tr>
<td>8</td>
<td>6</td>
<td>98±4.4</td>
<td>8.2±0.48</td>
<td>132±19</td>
<td>201±33</td>
</tr>
</tbody>
</table>

Mean 104±5.9(5.7%) 10.38±0.88(8.5%) 184±62(34%) 264±64(24%)  

Values on Lines 1–8 are means ± SD for each set of tests. The composite numbers represent the mean of the eight patients' values ± the average of the SD for each subject; the coefficient of variation is in parentheses. MAP = mean arterial blood pressure; [Na] = red cell Na content; Na Cotr = Na cotransport; K Cotr = K cotransport.
Results

The study subjects (Table 1) consisted of a population of black men, subdivided into three groups: 58 EH, 20 N~, and 12 N+. Of the 58 EH patients, 30 gave a positive family history (EH+) of hypertensive disease in a first-degree relative. The subjects in the EH group were on the average slightly older (mean age, 55 years) than those in the N~ group (mean age, 50 years, p > 0.05) or N+ group (mean, 48 years, p = 0.01). The average mean arterial blood pressure (MAP) was 117 mm Hg for the EH patients, 94 mm Hg for the N~ subjects (p < 0.001 vs EH), and 99 mm Hg for the N+ group (p < 0.001 vs EH). The small MAP difference between the N~ and N+ subjects was not significant (p > 0.05).

Red Blood Cell Sodium Content

The RBC Na content [Na], is a highly reproducible measurement. In the subgroup of eight subjects with multiple determinations over time (mean time, 4.3 months), the mean [Na], was 10.15 mmol/liter RBC and the average SD = 0.84 or 8.3% (Table 2).

There were differences in [Na], (mmol/liter RBC ± SD) between the EH and N~ groups (9.66 ± 3.02 vs 7.96 ± 1.97, p < 0.05) as well as between the N+ and N~ groups (10.38 ± 3.18 vs 7.96 ± 1.97, p < 0.01; Figure 2). The difference between the EH and N~ subjects was not significant. When the 30 EH+ patients were compared to the N~ subjects, the difference in [Na], increased (10.24 ± 3.23 vs 7.96 ± 1.97, p < 0.01). Despite the significant differences between the EH and N~ groups, when the individual MAPs from subjects in each category (EH, N+, and N~; n = 88) were plotted against the [Na], values (Figure 3), no linear correlation was found (r = 0.095, p > 0.1).

Table 3. Red Cell Cation Flux Parameters

<table>
<thead>
<tr>
<th>Cation parameters</th>
<th>EH (n = 53)</th>
<th>N+ (n = 12)</th>
<th>N+ (n = 20)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na cotransport (µmol/liter RBC/hr)</td>
<td>154±123</td>
<td>167±93</td>
<td>207±142</td>
<td>NS</td>
</tr>
<tr>
<td>K cotransport (µmol/liter RBC/hr)</td>
<td>200±103</td>
<td>215±115</td>
<td>254±136</td>
<td>NS</td>
</tr>
<tr>
<td>Na/K cotransport (ratio)</td>
<td>0.66±0.41</td>
<td>0.91±0.58</td>
<td>0.62±0.28</td>
<td>NS</td>
</tr>
<tr>
<td>Na permeability (rate coefficient)</td>
<td>0.026±0.004</td>
<td>0.025±0.005</td>
<td>0.024±0.003</td>
<td>NS</td>
</tr>
<tr>
<td>K permeability (rate coefficient)</td>
<td>0.021±0.007</td>
<td>0.019±0.004</td>
<td>0.019±0.006</td>
<td>NS</td>
</tr>
<tr>
<td>[Na], after loading (mmol/liter RBC)</td>
<td>41.3±2.2</td>
<td>40.3±2.1</td>
<td>40.9±2.5</td>
<td>NS</td>
</tr>
<tr>
<td>[K], after loading (mmol/liter RBC)</td>
<td>17.5±4.1</td>
<td>17.4±2.9</td>
<td>19.2±4.4</td>
<td>NS</td>
</tr>
</tbody>
</table>

Data are expressed as means ± SD. NS = not significant, p > 0.1.
A sampling of serum K concentrations was performed on 30 EH patients to assure that their K levels were normal when off antihypertensive therapy. The mean serum K was 4.2 ± 0.4 mEq/liter, and the mean [Na], for this subgroup equalled 9.62 ± 3.0 mmol/liter RBC. Regression analysis showed no correlation (r = 0.07, p = 0.7) between individual serum K and [Na]. Serum K levels were not measured in our control patients but were assumed to be normal.

**Sodium and Potassium Cotransport**

The mean furosemide-sensitive Na and K efflux rates (mmol/liter RBC/hr ± SD) are displayed in Table 3. Although there was a trend toward decreasing mean cotransport activity for the N", N", and EH groups (Na cotransport: 207 ± 142, 167 ± 93, 154 ± 123, mmol/liter RBC/hr, respectively), no statistical difference was demonstrated between the EH and N groups (p > 0.01). Selection for EH" did not significantly change the mean cotransport activity rate. The Na- and K-loading characteristics, cation permeability, and the furosemide-sensitive Na-to-K efflux ratio were similar for the three groups (for all values, r > 0.1). No linear relationship could be identified between MAP and furosemide-sensitive Na efflux (for linear regression, r = -0.023, p > 0.1). Furthermore, furosemide-sensitive Na efflux and [Na], did not demonstrate a significant linear relationship (r = 0.16, p > 0.1).

The changes over time in cotransport activity for an individual was also studied by analysis of variance. The eight individuals who underwent multiple assays (mean number of assays, 6.4) displayed marked individual variation (Table 2). The average coefficients of variation for Na and K cotransport were 34% and 24%, respectively. Certain individuals, however, showed little variation in their cotransport activity, while others demonstrated enormous fluctuation over time (Figure 4). Many of these assays were performed together in batches and were from subjects with both large fluctuations and little variation. This made it improbable that the large coefficients of variation encountered in some individuals were related to technical factors alone.

Cell swelling was not continuously monitored in our study. However, subsequent studies were performed in our laboratory with the use of the hemoglobin concentration method to determine changes in water content in 11 individuals. The RBCs were processed with the same protocol and techniques as described earlier. The cation loading (mean [Na], = 42 mmol/liter RBC, and mean [K], = 18 mmol/liter RBC) and cell permeability rate coefficients (mean kNa = 0.023 hr⁻¹ and mean kK = 0.015 hr⁻¹) were similar to those of the study subjects (Table 3). Based on an assumed water content of 65% for the fresh red cells, the mean increase in cell water content was 0.8% ± 1.8% (range = -2.3% to +3.5%).

**Discussion**

**Sodium and Potassium Cotransport**

The erythrocyte Na + K cotransport system was first described by Wiley and Cooper in 1974 as a ouabain-sensitive transport process that facilitates the inward cotransport of Na and K ions. This process, which is inhibited by furosemide, can mediate either efflux or influx under appropriate conditions. Dagher and Garay proposed that the activity of the RBC Na + K cotransport was significantly reduced in individuals with EH and their offspring. Their subjects included a French population of varying age, race, and sex. This finding has been confirmed, but with a variable degree of overlapping values. Other investigators did not use the pCMBS loading technique, and have not shown a significant reduction in cotransport activity between EH and N" populations. In contrast, Adragna et al., using pCMBS loading, demonstrated a significantly higher cotransport activity in their hypertensive subjects compared to normotensive controls. Methodology, demographic or racial differences between subjects, and small study sizes may all contribute to these discrepancies.

There has been no investigation of the relationship of RBC cotransport activity to EH in a population of adult black men. In a small subgroup of a larger interracial study of both males and females, Davidson et al. could not demonstrate a significant difference between black EH and N" controls. Garay et al., in a study performed on a black population from the Ivory Coast of Africa, also could not distinguish a difference in RBC cotransport between patients with EH and a group of normotensive individuals unslected for fami-
ily history. They also noted that the average cotransport activity for this population was markedly less than that encountered in their French subjects. 

Our present study indicates that erythrocyte cotransport activity is not a useful tool for the identification of EH in blacks because of the large overlap in values and individual fluctuations. The mean furosemide-sensitive efflux among the patients with EH was 154 ± 123 fmol/liter RBC/hr and 207 ± 142 for the N" subjects. Although the mean efflux rate for the EH group appeared lower, the differences was not significant (p > 0.1). Analysis of variance of a subgroup of subjects showed that fluctuations in cotransport activity for most individuals were large compared to a relatively constant [Na]. The physiological significance of these fluctuations is unclear, since it has been demonstrated that under normal conditions the RBC cotransport system does not affect a net Na flux. It is therefore not surprising that no relationship was demonstrated between the [Na], and the furosemide-sensitive Na efflux.

The absolute RBC furosemide-sensitive Na and K effluxes observed in the present report is lower, overall, than that reported by others. A number of factors may contribute to this observation. First, the actual intracellular cation concentrations after loading differ from those of other investigators. Dagher and Garay loaded their RBCs to a mean of 25 and 37 mmol/liter RBC and Adragna et al. to 50 and 61 mmol/liter RBC for [Na], and [K],, respectively. In our present study, mean [Na], and [K],, after loading were 41 and 18 mmol/liter RBC, respectively. It has been demonstrated that cotransport efflux rates are very sensitive to internal cation concentrations. Even though the internal cation concentrations were above the K values reported for both Na and K, lower internal cation concentrations, in part, may explain the lower cotransport rates obtained. However, this should not serve as an explanation for the lack of difference between the EH and N" subjects. The cation concentrations for both groups were virtually identical after loading (Table 3). Although the cotransport system has been reported to mediate Na + K flux in a 1:1 ratio, the average ratio in our study was 0:69. This ratio is in close agreement with that obtained by Swarts et al.

The low [K], after loading cannot explain the diminished Na-to-K flux ratios (Table 3), because a low [K], would, if anything, reduce the furosemide-sensitive K flux and thereby raise the Na-to-K flux ratio.

Adragna et al. have demonstrated that RBC swelling after cation loading can reduce cotransport activity. Although cell volume was not measured to our study, the changes in RBC water content was recorded in 11 randomly selected subjects. The range in RBC swelling extended to a maximum of +3.5%. This increase might lead to an artifactual depression in cotransport activity. However, with a mean increase in cell water content of +0.8% ± 1.8%, the large majority of RBCs studied should not have had depressed furosemide-sensitive cation flux on the sole basis of cell swelling.

Finally, cotransport activity may be depressed in black compared to white populations. Garay et al. found that furosemide-sensitive Na and K efflux rates were generally lower in cells from an Ivory Coast population than the efflux rates in their previously reported Parisian population. This may either be an effect of a lower K or a lower V in RBCs from black subjects. We have not, however, performed the necessary kinetic assays nor studied enough white subjects to provide additional data.

**Content of Sodium Red Blood Cells**

Differences in intraerythrocytic and intralymphocytic Na have previously been demonstrated between EH and N" subjects. In our current study, there was a significant difference in [Na], between EH and N" groups, but the overlap of values was considerable. We found that 70% of the EH group and 30% of N" individuals had [Na], values greater than 8.5 mmol/liter RBC. The individual measurements (Figure 2) demonstrated that the [Na], in the EH population was skewed toward higher values, but, because of the large overlap, this assay is not useful as a diagnostic tool. Inaccurate family history information could account for the few N" subjects with relatively high [Na],, but this reasoning cannot explain the low [Na], observed in some patients with EH.

The relationship between serum K levels and RBC [Na], for an individual is well known; as serum K falls, [Na], rises. A correlation between these two factors, however, has not been demonstrated on a population basis. Since the serum K was normal in a representative group of EH patients, it is unlikely that serum K levels played a significant role in the elevation of [Na], noted in our EH population relative to the N" controls.

Despite the elevated mean [Na], in the EH patients, we found no correlation between [Na], and individual blood pressures. It has been postulated that a rise in vascular smooth muscle [Na], leads to elevated [Ca], and subsequently to increased vascular tone. However, RBC [Na], may not necessarily be directly correlated with [Na], in other types of cells due to differences in membrane properties and also because of the ability of the hematopoetic system to increase the number of Na + K ATPase pumps per RBC in response to pump inhibition or alterations of extracellular cation concentrations.

In conclusion, our data suggests that there is a relationship between [Na], and EH in adult black males. However, a significant correlation was not shown between RBC cotransport and EH in this group, unlike some studies performed on white populations. Measurements of the RBC Na content and furosemide-sensitive Na and K efflux do not provide a reliable method for detecting individuals in a black population at risk for EH.

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