Red Blood Cell Na\(^+\),K\(^+\)-ATPase in Men with Newly Diagnosed or Previously Treated Essential Hypertension

RICHARD E. RINGEL, JOHN M. HAMLYN, BRUCE P. HAMILTON, GERARD A. PINKAS, STUART A. CHALEW, AND MICHAEL A. BERMAN

With the technical assistance of Richard Pavlis and Gregory Kuzbida

SUMMARY

Alterations of cellular function of Na\(^+\),K\(^+\)-adenosine triphosphatase (ATPase; Na\(^+\)-K\(^+\) pump) have been implicated in the pathophysiology of essential hypertension. Therefore, this aspect of red blood cell (RBC) Na metabolism was studied in black men with newly diagnosed, untreated essential hypertension (NEH) and a normotensive control group. RBC Na content, Na\(^+\)-K\(^+\) pump number (ouabain binding sites), and pump activity were measured. No statistically significant differences were found between the two groups for any of these three parameters. However, a group of previously treated essential hypertensive subjects (PEH) who had been withdrawn from therapy in the preceding 6 weeks were also studied. This group differed significantly from the NEH subjects in regard to all RBC Na\(^+\)-K\(^+\) pump parameters. Their RBC Na content (10.27 ± 3.23 vs 7.77 ± 2.52 mmol Na/L RBC; \(p = 0.006\)) was higher, and their Na\(^+\)-K\(^+\) pump activity (166 ± 50 vs 221 ± 87 nmol inorganic phosphate/mg membrane protein/hr; \(p = 0.03\)) and Na\(^+\)-K\(^+\) pump number (213 ± 40 vs 284 ± 85 binding sites/RBC; \(p = 0.001\)) were lower compared with those in NEH subjects. Although the PEH subjects were older and somewhat less hypertensive than their NEH counterparts, these factors were not found to influence the Na\(^+\)-K\(^+\) pump parameters. These results indicate that chronic diuretic therapy of patients with essential hypertension is associated with a reduced number of RBC Na\(^+\)-K\(^+\) pumps. Since RBCs are not considered target cells for diuretics, the effects of these drugs on RBC electrolyte metabolism may occur at the time of erythropoiesis by the production of RBCs with fewer Na\(^+\)-K\(^+\) pumps. Furthermore, the finding of elevated RBC Na content in PEH subjects acutely withdrawn from diuretic therapy is compatible with the renewed production of an endogenous natriuretic hormone that functions as a Na\(^+\)-K\(^+\) pump inhibitor.

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KEY WORDS • diuretics • essential hypertension • sodium • Na\(^+\),K\(^+\)-adenosine triphosphatase • ouabain

MUCH evidence has accumulated implicating alterations of cellular Na metabolism in the development of essential hypertension (EH).\(^1\) These abnormalities may be related to increased levels of a circulating inhibitor of Na\(^+\),K\(^+\)-adenosine triphosphatase (ATPase; Na\(^+\)-K\(^+\) pump) in patients with EH. It has been proposed that this substance primarily functions as a natriuretic hormone and that certain persons require higher levels of the inhibitor because of an inherited inability of their kidneys to excrete the large amounts of salt ingested in most modern societies.\(^1,2\) Increased levels of Na\(^+\)-K\(^+\) pump inhibition have been detected by a number of techniques\(^3\) in patients with EH. Demonstration of in vivo effects of pump inhibition on cellular Na metabolism, however, has been less definitive. Both increased and decreased red blood cell (RBC) Na\(^+\),K\(^+\)-ATPase activity have been described in persons with EH.\(^3\) Although many studies have shown a significant elevation of
RBC sodium content ([Na]) in EH, the overlap in values is usually substantial.

RBCs, as a population, are capable of escaping the effects of Na⁺-K⁺ pump inhibition following digitalization by increasing cellular pump density. This up-regulation phenomenon probably occurs during erythropoiesis, since circulating RBCs lack the ability to synthesize new proteins. In addition, the up-regulation phenomenon may obscure the difference between RBC [Na], Na⁺,K⁺-ATPase activity in hypertensive and normotensive (NT) groups. Therefore, we have measured [Na], Na⁺,K⁺-ATPase activity, and Na⁺-K⁺ pump number (ouabain binding sites) in RBCs from black men with normal blood pressure (BP), newly diagnosed EH (NEH), and previously treated EH (PEH). The PEH subjects had discontinued their medications within the preceding 6 weeks.

Subjects and Methods
Hypertensive Subjects
Subjects with EH were diagnosed and followed at the Hypertension Clinic of the Loch Raven Veterans Administration Medical Center, Baltimore, Maryland. All were black men older than 30 years of age with no other systemic illnesses. Criteria for diagnosis and inclusion in the study required systolic BP readings of 145 mm Hg or higher and diastolic BP readings of 95 mm Hg or higher on three or more visits. Chest radiograph; levels of serum electrolytes, blood urea nitrogen, and creatinine; and urinalysis results also were normal. Both NEH (untreated) and PEH subjects were included. Subjects whose hypertension was controlled by antihypertensive medication within the previous 6 months were taken off therapy 2 to 6 weeks before study and were hypertensive at the time of blood collection. 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 0930 and 1100. No special instructions or diets were given. With subjects in the sitting position, BP was measured prior to blood sampling.

Controls
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, without uremia or other systemic illnesses, and without digoxin or other medication intake. Criteria for inclusion were a systolic BP below 140 mm Hg and a diastolic BP below 90 mm Hg. 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, without uremia or other systemic illnesses, and without digoxin or other medication intake. Criteria for inclusion were a systolic BP below 140 mm Hg and a diastolic BP below 90 mm Hg. Blood samples were collected in the morning between 0830 and 1100. No special instructions or diets were requested. With subjects in the sitting position, BP was obtained before venipuncture.

Venipuncture
Thirty milliliters of blood was withdrawn directly into heparinized glass tubes (Vacutainer A3200 KA; Becton-Dickinson, Rutherford, NJ, USA) and maintained at room temperature until cell preparation was begun within 4 hours of collection. 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 American Chemical Society grade or better.

RBC Na Content
RBCs were separated from heparinized blood samples by centrifugation at 1700 g for 5 minutes. Plasma was removed, and the buffy coat discarded. 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 hemocytometer 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. The resulting solution was diluted without removing the membranes, and the Na and K concentrations were measured by atomic absorption spectrophotometry (Model AAS 457; Instrumentation Laboratories, Lexington, MA, USA). To suppress ionization, 25 mM CsCl was included in samples for Na determinations. The intracellular concentrations were then calculated and expressed in millimoles per liter of RBCs. No correction for extracellular trapped volume was made.

Na⁺,K⁺-ATPase Activity
After overnight storage at 4°C, RBCs were separated from plasma and buffy coat by centrifugation and washed in 0.9% saline. The cells were lysed in 10 volumes of 1 mM EDTA/Tris (pH 7.6) and centrifuged at 50,000 g for 15 minutes. Membrane washing was repeated until the membranes were hemoglobin-free, typically after four washes. Membranes were suspended in the EDTA/Tris solution. A concentrated stock solution containing Na, adenosine 5'-triphosphate (ATP), Mg, and K was added to 1.6 ml of membrane suspension. This final retention suspension (volume, 1.76 ml) does not promote displacement of ATPase inhibitors from Na⁺-K⁺ pump sites and contains 100 mM NaCl, 20 mM KCl, 3 mM MgCl₂, 2 mM Na₄ATP, 0.9 mM EDTA/Tris, and 15 mM Tris HCl, pH 7.6. The suspensions were incubated at 37°C for 40 minutes and then cooled in an ice water slush. Then 200 μl was transferred (in quadruplicate) from each suspension into media containing 100 mM NaCl, 20 mM KCl, 3 mM MgCl₂, 2 mM Na₄ATP, 1 mM EDTA, 80 mM Tris HCl, pH 7.6, 37°C for Na⁺,K⁺-ATPase assay.

Ouabain-insensitive ATPase activity was determined in the same assay medium containing 0.1 mM ouabain in the absence of NaCl and KCl. Two hundred microliters was transferred (in quadruplicate) into assay medium containing 1.0 ml of 10% sodium dodecyl sulfate (SDS) to measure background phosphate levels.
(blanks). The assay suspensions (in quadruplicate) were incubated at 37°C for 30 minutes, reactions were terminated by adding 1.0 ml of 10% SDS to each suspension (except blanks), and all samples were cooled in an ice water bath. Inorganic phosphate (P) release was determined colorimetrically at 720 nm. After subtracting background P, ATPase activity was expressed as nanomoles of P per milligram of membrane protein per hour. Membrane protein content was estimated by the method of Lowry et al. using bovine serum albumin as a standard.

Fifteen subjects and nonstudy volunteers had second assays performed under stable clinical conditions to analyze the reproducibility of the activity measurements. Linear regression analysis of the first versus second assay results indicated a correlation coefficient of 0.45 (p < 0.1). If three subjects who were studied early in our experience and demonstrated poor reproducibility were excluded from the reproducibility analysis, the correlation coefficient for the remaining 12 paired assays became 0.87 (p < 0.001). Nevertheless, because of the high variability detected, five NT subjects underwent multiple RBC ATPase assays during the course of the study. All had three or more determinations (mean, 6.4; range, 3–18) over 2 to 18 months (mean, 8.6 months). The average, relative standard deviation (RSD) was found to be 17.9 ± 10.4%.

Na⁺-K⁺ Pump Number

After overnight storage at 4°C, RBCs were separated from plasma and buffy coat by centrifugation and washed twice in 0.9% saline solution. A suspension containing 400 μl of RBCs was centrifuged in duplicate at 1500 g for 7.5 minutes, and the supernatant was aspirated. The cells were resuspended in 8.0 ml of an iced solution containing 10 mM MgCl₂, 5 mM glucose, 1 mM Na₂HPO₄, 1 mM HEPES (adjusted to pH 7.4 with Tris base), and ouabain, either 10⁻⁷ M (specific activity, 6.16 Ci/mmol: Solution A) or 10⁻⁸ M (specific activity, 6.16 mCi/mmol: Solution B). Solution A was used for specific binding of ouabain to Na⁺-K⁺ pump sites, and Solution B, with its excess of cold (unlabeled) ouabain, was used for nonspecific membrane binding. The lysed cells were vortexed and incubated at 37°C for 2.5 hours (equilibrium binding). The membrane ghosts were then cooled, and a volume equal to 50 μl of RBCs was collected using a Brandell cell harvester (Gaithersburg, MD, USA). Trapped membranes were collected using Schleicher and Schnell wetted No. 25 glass filters (Keene, NH, USA). The filters were washed with Solution B and placed in scintillation vials with 0.5 ml of a 10% SDS solution. Then 10 ml of liquid scintillation medium (Liquiscint) was added, after which the tubes were vigorously vortexed. The primary advantages of this method are that 1) cells are not rinsed with peroxide to remove the hemoglobin that interferes with scintillation counting and 2) the number of RBCs being assayed is known and subject to little variation, thus allowing for reproducible determination of binding sites per cell. This procedure also produces a physiological K concentration of 4.2 to 4.6 mM in the lysate/binding medium. Experiments performed with various K concentrations showed that, relative to a zero K medium, [³H]ouabain binding was 93.5 ± 1 and 95.5 ± 0.8% complete (n = 4) in 4.5 mM K and 3.5 mM K media, respectively.

Scintillations were counted on a Packard Tricarb 460c counter (Rockville, MD, USA) using an external standard. The efficiency on ³H counting was typically 35%. All samples were quench-corrected. Background counts were measured using blank filters prepared in the same manner. Data are expressed as number of Na⁺-K⁺ pumps (ouabain binding sites) per RBC.

The number of Na⁺-K⁺ pumps (ouabain binding sites) per RBC was calculated by converting scintillation counts per minute (cpm) to disintegrations per minute (dpm) using a prepared efficiency (quench correction) curve and multiplying by a constant that is an expression of [³H]ouabain molecules/dpm × cell number. This constant is the product of Avogadro's number in molecules per mole divided by the dpm per mole for [³H]ouabain and further divided by the number of cells assayed. The cell number is obtained by multiplying 50 μl by 1.11 X 10¹³ cells/L using a mean corpuscular volume (MCV) of 90 fl/cell. Thus, the number of [³H]ouabain molecules bound to each cell membrane is derived.

The reproducibility of the [³H]ouabain binding technique was also tested. Fifteen subjects and nonstudy volunteers underwent repeat binding assays. Samples were obtained 0.5 to 13 months apart (mean, 3.5 months). Linear regression analysis demonstrated a correlation of 0.979 (p < 0.0001), indicating excellent reproducibility over time.

Statistics

The statistical significance of subject grouping, when mean arterial pressure (MAP) and age were controlled on each variable, was by analysis of variance. Preplanned contrasts between subject groups (NT vs NEH, PEH vs NEH, and NT vs PEH) are reported. Logarithmic transformation of data was performed before statistical analysis when needed to reduce the heterogeneity of variances. All data are expressed as the mean ± 1 SD. Statistical significance was considered at p values less than or equal to 0.05.

Results

The study included 33 NT and 27 NEH subjects. The NT subjects were, on average, younger than the NEH subjects (44 ± 11 vs 50 ± 10 years; p = 0.02). No differences (p = 0.1) were found between the NEH and NT groups for RBC [Na⁺], ratio of intracellular K ([K]i) to [Na⁺], ([K]i/[Na⁺]). Na⁺-K⁺-ATPase activity, or number of ouabain binding sites (Na⁺-K⁺ pumps) per RBC (Table 1). Age and MAP had no effect on these parameters (p > 0.1 for all comparisons). Plasma K concentrations were within normal for all subjects (4.0 ± 0.3 and 4.1 ± 0.4 mEq/L for NT and NEH subjects, respectively).

As expected, a highly significant relationship was demonstrated between [Na⁺] and the number of Na⁺-K⁺ pumps per RBC. When [Na⁺] was related to the
TABLE 1. Characteristics of Newly Diagnosed Essential Hypertensive, Normotensive, and Previously Treated Essential Hypertensive Subjects

<table>
<thead>
<tr>
<th>Variable</th>
<th>NEH</th>
<th>NT</th>
<th>PEH</th>
<th>P (NEH vs NT)</th>
<th>P (NT vs PEH)</th>
<th>P (NEH vs PEH)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yr)</td>
<td>50±10 (27)</td>
<td>44±11 (33)</td>
<td>58±8 (21)</td>
<td>0.0001</td>
<td></td>
<td>0.003</td>
</tr>
<tr>
<td>MAP (mm Hg)</td>
<td>125±11 (27)</td>
<td>96±6 (33)</td>
<td>119±6 (21)</td>
<td>0.0001</td>
<td></td>
<td>0.04</td>
</tr>
<tr>
<td>[Na]j (mmol/L RBC)</td>
<td>7.77±2.52 (25)</td>
<td>7.97±2.16 (30)</td>
<td>10.27±3.23 (19)</td>
<td>NS</td>
<td>NS</td>
<td>0.006</td>
</tr>
<tr>
<td>[K]j (mmol/L RBC)</td>
<td>99.12±4.92 (25)</td>
<td>97.07±4.08 (30)</td>
<td>98.63±3.95 (19)</td>
<td>NS</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>[K]j/[Na]j</td>
<td>14.02±4.48 (25)</td>
<td>13.19±4.15 (30)</td>
<td>10.61±3.61 (19)</td>
<td>0.04</td>
<td>NS</td>
<td>0.01</td>
</tr>
<tr>
<td>Na+-K+ pump number</td>
<td>284±85 (24)</td>
<td>NS</td>
<td>221±87 (16)</td>
<td>0.06</td>
<td>166±50 (13)</td>
<td>0.03</td>
</tr>
<tr>
<td>Na+-K+ pump activity</td>
<td></td>
<td>NS</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

All values are means ± 1 SD. Number of subjects is shown in parentheses.
NEH = newly diagnosed essential hypertension; NT = normotension; PEH = previously treated essential hypertension.

inverse of the number of Na+-K+ pumps per RBC (Na+-K+ pump number-1), a linear relationship was found ($r = 0.837, p = 0.0001$; Figure 1). A strong linear relationship was seen between the ratio of [K]j to [Na]j, and the number of Na+-K+ pumps per RBC ($r = 0.827, p = 0.0001$). Significant linear correlations were also present for [Na]j, ratio of [K]j to [Na]j, and Na+-K+ pump number versus Na+-K+ pump number (Figure 2), ratio of [K]j to [Na]j, and Na+-K+ pump number versus Na+-K+ pump number (Figure 1), ratio of [K]j to [Na]j, and Na+-K+ pump number versus Na+-K+ pump number (Figure 2). The relationships were not affected by subject grouping (i.e., whether analyzed for NT or NEH groups), as illustrated in Figures 1 and 2.

The study also included a group of 21 PEH subjects (see Table 1). Hypertension in these subjects had been controlled in the preceding 6 months, but medications were withdrawn 2 to 6 weeks before blood sampling. Therapy had included diuretics alone (hydrochlorothiazide with or without spironolactone; $n = 13$) or in combination with β-blocking agents ($n = 8$). No PEH subjects were hypokalemic (plasma K ≤ 3.5 mEq/L) at the time of this study (mean K level, 4.0 ± 0.3 mEq/L) nor during the course of their prior treatment. The PEH subjects were older ($58 ± 8$ vs $50 ± 10$ years; $p = 0.003$) and less hypertensive (MAP: $119 ± 6$ vs $125 ± 11$ mm Hg; $p = 0.04$) than the NEH subjects. Significant differences between the PEH and NEH groups were found for RBC Na$^+$ (10.27 ± 3.23 vs 7.77 ± 2.52 mmol/L RBC; $p = 0.006$), Na+-K+ pump number (213 ± 40 vs 284 ± 85 ouabain binding sites/RBC; $p = 0.001$), and Na+-K+ pump activity (166 ± 50 vs 221 ± 87 nmol Pj/mg protein/hr; $p = 0.03$; see Table 1) even when age and MAP were statistically controlled.

RBC Na metabolic parameters were also compared between the NT and PEH groups. The PEH subjects were older than the NT controls ($58 ± 8$ vs $44 ± 11$ years; $p = 0.0001$), but when age was controlled, it was not found to affect statistical comparisons. Intraerythrocytic [Na]j was higher in the PEH group ($10.27 ± 3.23$ vs $7.97 ± 2.16$ mmol/L RBC; $p = 0.01$). The ratio of [K]j to [Na]j, ($10.61 ± 3.61$ vs $13.19 ± 4.15$; $p = 0.006$).

**FIGURE 1.** Linear relationship between the inverse of the number of RBC Na+-K+ pumps and the RBC Na content was identical for both normotensive (NT) and newly diagnosed essential hypertensive (NEH) subjects.

**FIGURE 2.** RBC Na+-K+ pump activity and Na content were linearly related without dependence on BP status (e.g., normotension [NT] or newly diagnosed essential hypertension [NEH]). $P_i$ = inorganic phosphate.
RED BLOOD CELL NA⁺,K⁺-ATPASE IN ESSENTIAL HYPERTENSION/Ringel et al.

Discussion

The results of this investigation demonstrate that RBC Na metabolism in subjects with EH previously treated with diuretics is significantly different from that of untreated, hypertensive subjects. Diuretic therapy appears to produce a decrease in RBC Na⁺-K⁺ pump number. Furthermore, the [Na⁺], content was higher in PEH subjects than in NEH subjects. These differences must be considered when studying the Na metabolism of RBCs from EH subjects recently withdrawn from therapy. Such data will not accurately reflect the underlying physiology of EH.

A number of techniques for quantitating RBC (Na⁺, K⁺-ATPase activity have been developed. We chose to employ the classic colorimetric method of measuring the Pᵢ released during ATP hydrolysis in the presence of prepared RBC membranes. Repetitive determinations performed during the study had an average coefficient of variation of 17.9%. This degree of intrapatient variation accounts for much of the scatter in the relationship between RBC Na⁺-K⁺ pump number and activity (r = 0.52) as well as between [Na⁺] and Na⁺-K⁺ pump activity (r = 0.45). Nevertheless, this variation does not negate the conclusions drawn from comparisons of the three study groups. The three subject groups were studied randomly during the project period, thus virtually eliminating biases that may be introduced by alterations in laboratory technique. The activity levels reported should be viewed primarily as a relative index of Na⁺-K⁺ pump function in the three study groups. The nature of the method does not allow the data to be interpreted as a quantitative measure of maximal RBC Na⁺-K⁺ pump activity.

Measurement of Na⁺-K⁺ pump number by [³H]ouabain binding is highly reproducible, as indicated in the Methods section. It must be noted, however, that the actual number of pumps per cell may not be comparable among different laboratories. Many factors probably contribute to this variability. The number of sites per cell achieved depends on whether whole RBCs or cell membranes are used for [³H]ouabain binding. More sites are available for binding on whole cells than on membranes, which fold and may reseal inside-out. With our method of lysing RBCs in the ouabain incubation, a physiological K concentration results that can reduce ouabain binding by approximately 6% (see Methods). Furthermore, the number of cells contained in each assay aliquot was based on an average MCV of 95 fl/cell. Subgroups of subjects from each population category had RBC MCV measurements performed; MCV was 94 for the NT group (n = 22), 97 for the NEH group (n = 16), and 92 fl/cell for the PEH group (n = 12). At most, this method underestimates [³H]ouabain binding by 7%. The average number of specific ouabain binding (Na⁺-K⁺ pump) sites per RBC for our NT population was estimated at 254/cell. This value falls within the wide range of reported values for RBC cardiac glycoside binding, which extends from approximately 200 to 1200 molecules bound/cell.

The relationship between EH and RBC [Na⁺], has been under investigation for almost 30 years. Results have been conflicting. Most smaller studies have not revealed differences between subjects with EH and those with normal blood pressure. However, very large, tightly controlled studies do demonstrate significantly elevated RBC [Na⁺], in patients with EH. One such study involved 100 newly diagnosed hypertensive and 908 NT Nigerians. A difference was shown for RBC [Na⁺], between the two groups (13.6 ± 0.59 [SEM] vs 9.7 ± 0.13 mmol/L RBC; p<0.001), but the overlap between them was large (EH range, 3.6-31.3 mmol/L RBC; control range, 2.4-28.2 mmol/L RBC). Thus, it is easy to understand why significant differences might not be found in smaller studies.

No difference in RBC [Na⁺], was demonstrated between the NEH subjects and NT controls in our relatively small, black, inner-city population. Nevertheless, in a previous report we described a small but significant [Na⁺], elevation (9.66 ± 3.02 vs 7.96 ± 1.97 mmol/L RBC) in subjects with EH as compared with NT controls. However, all the EH subjects had been treated with antihypertensive medications and had been withdrawn from therapy 2 to 6 weeks before entering the study. Therefore, these subjects were more similar to the currently described PEH subjects, who had significantly higher RBC [Na⁺], levels than their NT controls (10.27 ± 3.23 vs 7.97 ± 2.16 mmol/L RBC; p = 0.01).

The apparent cause of the RBC [Na⁺], elevation in PEH subjects is a reduced number of Na⁺-K⁺ pumps per RBC. As in other reports, we have shown a close correlation between RBC [Na⁺] and Na⁺-K⁺ pump number (see Figure 1). The strength of this relationship reaffirms the dominant role played by membrane Na⁺,K⁺-ATPase in regulating [Na⁺].

The central question, however, is why the PEH subjects, relative to the NEH group, should have had 25% fewer Na⁺-K⁺ pumps/RBC (213 vs 284), 25% lower Na⁺-K⁺ pump activity (166 vs 221 nmol P₃/mg protein/hr), and 33% higher [Na⁺] (10.27 vs 7.77 mmol/L RBC). The following explanation is proposed: Levels of a circulating Na⁺,K⁺-ATPase inhibitor gradually rise as part of the pathophysiological process eventually resulting in EH. The hematopoietic system responds to the Na⁺-K⁺ pump inhibition by increasing the number of Na⁺-K⁺ pumps on newly formed RBCs. Thus, the two forces (endogenous pump inhibition and Na⁺-K⁺ pump number) remain in balance and act to maintain relatively normal RBC [Na⁺]. The absence of a significant difference in Na⁺-K⁺ pump number between the NEH and NT groups has two alternative explanations consistent with the proposed theoretical considerations. First, it is possible that the endogenous inhibitor does not dissociate from all Na⁺-K⁺ pump
sites and competes with the [$^3$H]ouabain for binding to the RBC membranes, thus reducing the number of sites counted in the NEH subjects. Second, EH subjects may begin with a lower than normal number of RBC Na$^+$-K$^+$ pumps. This possibility is supported by the demonstration that PEH subjects have fewer Na$^+$-K$^+$ pumps than the NT controls (213 vs 254 Na$^+$-K$^+$ pumps/RBC, $p = 0.05$), suggesting that the reduction in level of natriuretic hormone per Na$^+$-K$^+$ pump inhibitor allows the hematopoietic system to produce RBCs with a basal number of pumps.

The proposed role for the described endogenous pump inhibitor is that of a natriuretic agent. $^{1, 19}$ When EH is treated with diuretic (natriuretic) medications, the stimulus for production of this "hormone" is presumably reduced. With less inhibition the Na$^+$-K$^+$ pumps might be able to decrease RBC [Na]. Evidence for this effect has been presented by the demonstration of a fall in RBC [Na], in hypertensive subjects treated with hydrochlorothiazide. $^{16}$ During diuretic therapy of EH, withdrawal of endogenous Na$^+$,K$^+$-ATPase inhibition from the hematopoietic system might allow it to generate new cells with fewer functioning RBC Na$^+$-K$^+$ pumps. If diuretics are discontinued after chronic therapy, levels of the natriuretic (Na$^+$-K$^+$ pump-inhibiting) hormone would be expected to rise again. However, in this acute situation the RBCs now have "too few" Na$^+$-K$^+$ pumps and cannot fully respond to the renewed pump inhibition. In this situation the RBC [Na] should rise until new RBCs with more pumps can be produced. This process appears to take many weeks, apparently coinciding with the 120-day RBC life span. $^9$

The strength of this hypothesis rests on two factors: the existence of a circulating Na$^+$,K$^+$-ATPase inhibitor that functions as a natriuretic agent and the ability of the hematopoietic system to respond to Na$^+$,K$^+$-ATPase inhibition by increasing the number of Na$^+$-K$^+$ pumps per RBC. Plasma extracts from hypertensive animals and humans can produce ATPase inhibition. Furthermore, natriuretic substances have been found in the blood and urine of animals and humans after volume expansion and increased salt intake. $^{2, 17-19}$ However, only indirect evidence is available suggesting that a single factor can produce both of these effects. $^{20, 21}$ Direct confirmation awaits identification and purification of a substance with both capabilities.

The hematopoietic system has been shown to have the ability to respond to Na$^+$,K$^+$-ATPase inhibition following digitalization by increasing the number of Na$^+$-K$^+$ pumps per RBC. $^{4, 5}$ Na$^+$-K$^+$ pump inhibition caused by digoxin therapy is followed by a transient rise in RBC [Na], and subsequently by a rectification, bringing the level back to normal. $^{1, 22}$ A number of studies have demonstrated increased RBC Na$^+$,K$^+$-ATPase activity and pump number in response to digoxin therapy. One such study showed that uptake of $^{86}$Rubidium by RBCs (a measure of Na$^+$,K$^+$-ATPase activity) fell with initiation of digoxin treatment and returned to normal after 2 months of therapy. $^9$ Furthermore, [$^3$H]digoxin was used to measure the number of available binding (pump) sites before and after digoxin therapy. The number of available sites was found to decrease after treatment was started (due to sites already occupied by digoxin) but increased to control levels within 2 months. $^4$ In a similar set of experiments, other researchers showed that RBC Na$^+$,K$^+$-ATPase activity decreased initially after digitalization. Activity rose again during the chronic phase, increasing progressively with duration of therapy. In some persons, activity eventually exceeded control levels. $^{22}$ One possible explanation for this effect might be an attenuation of the membrane receptor's affinity for digoxin. However, it has been demonstrated that the receptor's dissociation constant for digoxin does not change during chronic therapy. $^{23}$ Furthermore, by measuring the number of available [$^3$H]ouabain binding sites, a small but significant ($p = 0.05$) increase was demonstrated in the number of Na$^+$-K$^+$ pumps per RBC in patients taking digoxin for 6 months compared with normal controls. Malini et al. $^5$ have also demonstrated increased numbers of digoxin receptors on RBC membranes from patients on a regimen of chronic digoxin therapy. Although this effect has been definitively demonstrated for the RBC, we may not be able to extrapolate these findings to other tissues. For instance, the number of [$^3$H]ouabain binding sites and the dissociation constant for ouabain binding in mammalian skeletal muscle decrease after chronic exposure to the Na$^+$,K$^+$-ATPase inhibitory effects of K depletion. $^{24}$

In conclusion, elevated levels of an endogenous Na$^+$,K$^+$-ATPase inhibitor may produce a rise in RBC Na$^+$-K$^+$ pump number in EH, but the effects are too subtle and occur over too long a time to be discerned adequately in small group studies. Nevertheless, when persons with EH are treated with diuretics, the stimulus to produce the inhibitor presumably is withdrawn and the number of Na$^+$-K$^+$ pumps per RBC falls. Thus, when treatment is discontinued and levels of the putative Na$^+$,K$^+$-ATPase inhibitor rise again, the RBCs have "too few" pumps to maintain the status quo and their RBC [Na], rises. Longitudinal studies of subjects before, during, and after diuretic treatment of EH are needed to confirm this work.

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References

1. MacGregor GA. Sodium is more important than calcium in essential hypertension. Hypertension 1985;7:628-637
4. Ford AR, Areson JK, Graeme-Smith DG, Carver JG. The acute changes seen in cardiac glycoside receptor sites, $^{86}$Rb uptake and intracellular sodium concentrations in the erythrocytes of patients during the early phases of digoxin therapy are
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