Reduced Sodium Concentration and Increased Sodium-Potassium Pump Activity of Erythrocytes in Human Hypertension

GEZA SIMON AND CAROL R. ENGEL

SUMMARY Erythrocyte Na, Na/K, and ouabain-sensitive and ouabain-insensitive 86Rb uptake (K transport) were measured in whole blood of 16 normotensive and 19 hypertensive white male subjects, within seconds or minutes after withdrawal of blood. Erythrocyte Na, and Na/K, were reduced (p<0.05), and ouabain-sensitive 86Rb uptake was increased (p<0.01) in hypertensive subjects. In a separate group of hypertensive white male subjects, an inverse correlation was found between erythrocyte Na/K and ouabain-binding sites per erythrocyte (r = 0.85, p<0.01, n = 9). The abnormalities of erythrocyte cation fluxes in hypertensive subjects are similar to those induced by aldosterone in vascular smooth muscle cells and by glucocorticoid administration in the erythrocytes of human subjects, suggesting similarities in pathogenesis. (Hypertension 9 [Suppl III]: 111-13—111-18, 1987)

KEY WORDS • intracellular sodium • potassium • rubidium-86 uptake • [3H]ouabain binding

ABNORMALITIES of all the major erythrocyte Na transport systems have been reported in human hypertension.1,2 With the exception of an increased Na-Li countertransport system, which under physiological conditions does not mediate any transport of Na across cell membranes, the results have been contradictory. Several investigators have reported increased activity of the ouabain-sensitive Na+-K+ pump, but others could not confirm this finding.2-5 The activity of the Na+-K+ cotransport system has been reported as reduced, increased, and unchanged.1,6 The net effect of these abnormalities in Na fluxes on the transmembrane Na gradient of red blood cells (RBCs) in human hypertension is uncertain. There have been reports of increased and unchanged erythrocyte Na content in human hypertension.7-9

The two main reasons for these contradictory findings appear to be the genetic heterogeneity of the disease and methodological problems.1,10,11 The genetic aspects of erythrocyte cation transport are under investigation in several laboratories.12,13 In our laboratory, we have concentrated on the methodological problems. The tests used by previous investigators required extensive in vitro manipulations and the separation of RBCs from plasma. It has been shown that measurements of cation contents and transport in nucleated cells are subject to in vitro influences of temperature, pH, and time of incubation.14 Separation of cells from plasma, which contains a multiplicity of cations, hormones, growth factors, and so on, may in itself bring about rapid changes in cation gradients in a manner that is analogous to the re-addition of serum or plasma to quiescent cells in an artificial medium.15,16 We have, therefore, devised techniques that allowed us to study RBCs under experimental circumstances that resembled in vivo conditions. Recently, we have published our measurements of erythrocyte Na and K concentrations (Na, and K,) in hypertensive and normotensive subjects.17 Samples for measurements were prepared within seconds after venipuncture by centrifuging whole blood over oil. This was possible because the white blood cells and platelets in the cell pellet had no detectable effect on the erythrocyte cation measurements.17 In the present study, we extend these investigations to the study of ouabain-sensitive and ouabain-insensitive erythrocyte cation fluxes in hypertensive and normotensive subjects. Techniques were developed to measure K transport within minutes after

From the Department of Medicine, Veterans Administration Medical Center and University of Minnesota, Minneapolis, Minnesota.
Supported by United States Public Health Service Grant HL34400.
Address for reprints: Geza Simon, M.D., 111C2, VA Medical Center, Minneapolis, MN 55417.
venipuncture without cell separation and in the subject’s own plasma. The relationship between erythrocyte cation concentration and pump activity was investigated by correlating erythrocyte Na/K, with the number of ouabain-binding sites per erythrocyte.

Materials and Methods
We studied white male patients with mild to moderate, uncomplicated hypertension. The presence of hypertension was documented by blood pressure measurements on three or more occasions. Secondary causes of hypertension were ruled out by clinical and laboratory examination. The laboratory tests included measurements of serum creatinine and potassium, 1-hour ambulatory plasma renin activity (PRA; radioimmunoassay of angiotensin I) 24-hour urinary creatinine clearance, and urinalysis. Intravenous pyelography or isotope renography was not routinely performed to rule out renovascular hypertension. Healthy normotensive subjects drawn from hospital and laboratory staff served as controls. All subjects were without antihypertensive or any other medication for at least 2 weeks prior to the study. At the time of blood drawing, all subjects had fasted for 3 hours or more. Blood was drawn from subjects twice, once for measurement of erythrocyte Na, and K, and once for measurement of erythrocyte 86Rb uptake. The protocol was approved by the institutional review board for human subject research.

Erythrocyte Sodium and Potassium Concentrations
Two-milliliter aliquots of heparinized venous blood were mixed with 20 μl of 125I-labeled serum albumin (Mallinckrodt, St. Louis, MO, USA). Each 100-μl sample of blood was layered over oil (density 1.05; Aldrich Chemical, Milwaukee, WI, USA) and centrifuged for 30 seconds at 13,000 g. Less than 60 seconds elapsed from the time of venipuncture to the time of separation of RBCs from plasma. RBC pellets were saved for measurement of total water content (by differential weighing after overnight heating at 104°C) and for radioactive counting. The supernatant plasma was counted in a liquid scintillation counter (Tri-carb Model 3375, Packard, Downers Grove, IL, USA). For counting, the cell pellets were digested in 0.5 ml of 1.0 N NaOH containing 0.01% octoxynol (Triton X) for 60 minutes at 50°C. The volume of digested cells was brought to 5 ml. To correct for quenching that was due to the pink color of the hemolysate, the supernatant plasma and the standards were also counted in hemolysate prepared from blank cell pellets. To the third tube (see above), 20 μl of 125I-albumin was added as soon as sampling of tubes 1 and 2 was completed. After mixing, 100-μl aliquots of supernatant plasma were centrifuged over oil for estimation of extracellular plasma trapping and measurement of water content, as outlined above. (Extracellular plasma trapping was also estimated with 38Co-EDTA, but these results are not reported here.) Calculation of 86Rb uptake was based on radioactive counts of cell pellets minus radioactivity in plasma multiplied by plasma trapping and water content of cell pellets. Results were expressed as μmol/L cell water · min⁻¹. The specific activity of 86Rb fluxes was calculated from the ratio of 86Rb activity (in cpm) in plasma and plasma K concentration. Ouabain-sensitive 86Rb uptake was measured by subtracting ouabain-insensitive uptake from the total uptake. Uptake of 86Rb was linear with time for up to 20 minutes (n = 3).

Ouabain-Binding Sites
In a separate group of nine hypertensive and 10 normotensive white male subjects, erythrocyte Na, was correlated with the number of [3H]ouabain-binding sites per erythrocyte. Measurements of 1-hour ambulatory PRA and 24-hour urinary creatinine clearance in these subjects were not performed. The procedures followed for ouabain binding were a modification of
those used by DeLuise et al.19 and were previously published from our institution.20 In our study, 20 to 25 ml of venous blood was drawn from fasting subjects into heparinized glass tubes. Erythrocyte Na, and K, were measured as described earlier. The remaining blood was centrifuged at room temperature for 5 minutes. Theuffy coat was discarded, and erythrocytes were washed three times with 15 ml of a 150-mM choline chloride solution and resuspended in buffer (pH 7.4; 140 mM NaCl, 30 mM HEPES, 10 mM dextrose) to a final cell concentration of 15 to 20%. Ouabain binding to RBCs was carried out in duplicate in 4-ml glass test tubes by adding 25 μl of unlabeled ouabain (total amounts of 1.25, 2.50, 6.25, and 12.50 pmol), 25 μl of [3H]ouabain (20 Ci/mmol; New England Nuclear) (total amounts of 1.25, 2.50, 6.25, and 12.50 pmol), and 200 μl of RBCs suspended in buffer. The final concentration of ouabain in the tubes was 10, 20, 50, and 100 nM. The tubes were covered and incubated in a 37°C shaking water bath for 3 hours. The RBCs were then rapidly washed three times with 1 ml of ice-cold choline chloride. The RBC membranes were extracted by adding 0.5 ml of a 5% solution of trichloroacetic acid, followed by mixing and centrifuging for 5 minutes. Supernatant solution (0.4 ml) was added to 10 ml of scintillation fluid (Biofluor, New England Nuclear) (total amounts of 1.25, 2.50, 6.25, and 12.50 pmol), and 200 μl of RBCs suspended in buffer. The concentration of ouabain in the tubes was 10, 20, 50, and 100 nM. The tubes were covered and incubated in a 37°C shaking water bath for 3 hours. The RBCs were then rapidly washed three times with 1 ml of ice-cold choline chloride. The RBC membranes were extracted by adding 0.5 ml of a 5% solution of trichloroacetic acid, followed by mixing and centrifuging for 5 minutes. Supernatant solution (0.4 ml) was added to 10 ml of scintillation fluid (Biofluor, New England Nuclear) and counted in a liquid scintillation counter as described for ^86Rb. Specifically bound [3H]ouabain was calculated by subtracting nonspecific counts (determined in the presence of 0.1 mM unlabeled ouabain). Nonspecific counts were always less than 5% of total counts. Scatchard plots were constructed for each subject to determine the concentration of binding sites in the final cell suspension (x-intercept). Data points were accepted if the linear correlation coefficient (r) was greater than 0.95. The x-intercept was then converted to the number of sites per RBC as follows:

\[
\text{sites/RBC} = \frac{x\text{-intercept} \times (6.02 \times 10^{23})}{\text{dilution factor} \times \text{concentration of RBC}}
\]

Concentration of erythrocytes in the cell suspension was measured in a Coulter counter.

The reported values are means ± SD. Comparison of measured parameters in the two groups was made by Student's t test for unpaired replicates. Linear regression analysis was used to correlate measurements obtained in the same group of subjects. The null hypothesis was rejected when the p value was less than 0.05 (two-tailed test).

**Results**

The clinical characteristics of hypertensive and normotensive subjects whose blood was used for measurements of erythrocyte Na, and K, and ^86Rb uptake are shown in Table 1. Six normotensive subjects had a family history of hypertension, and one subject's family history was unknown. All but seven of the hypertensive subjects had a family history of hypertension. One-hour ambulatory PRA exceeded 4.0 ng/ml·hr⁻¹ in three hypertensive subjects and was 0.5 or less in two. Twenty-four-hour urinary creatinine clearance was greater than 75 ml/min·(1.73 m²)⁻¹ in all but four subjects. The plasma Na and K concentrations of normotensive and hypertensive subjects were the same (data not shown). Erythrocyte Na, and Na/K, were reduced, and total and ouabain-sensitive ^86Rb uptake were increased in hypertensive subjects (Table 2 and Figure 1). The range of erythrocyte Na, in normotensive subjects was 13.6 to 20.8 mmol/L cell water and in hypertensive subjects 12.9 to 18.1. Ouabain-sensitive ^86Rb uptake by erythrocytes in normotensive subjects with a family history of hypertension was 24 ± 7 μmol/L cell water·min⁻¹ (n = 6) compared to 21 ± 4 in normotensive subjects without such history (n = 9, NS). The three highest values of ouabain-sensitive ^86Rb uptake in normotensive control group were registered in subjects with a family history of hypertension (see Figure 1). In hypertensive subjects with or without a family history of hypertension, ouabain-sensitive ^86Rb uptake of erythrocytes was the same (data not shown). The correlation coefficient between

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Normotensive subjects (n = 16)</th>
<th>Hypertensive subjects (n = 19)</th>
</tr>
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<tbody>
<tr>
<td>Na (mmol/L cell water)</td>
<td>16.9 ± 2.0</td>
<td>15.5 ± 1.6*</td>
</tr>
<tr>
<td>K (mmol/L cell water)</td>
<td>140 ± 6</td>
<td>142 ± 6</td>
</tr>
<tr>
<td>Na/K</td>
<td>0.120 ± 0.018</td>
<td>0.109 ± 0.012*</td>
</tr>
<tr>
<td>^86Rb uptake (μmol/L cell water·min⁻¹)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>38.8 ± 6.4</td>
<td>45.8 ± 8.5f</td>
</tr>
<tr>
<td>Ouabain-sensitive</td>
<td>22.6 ± 5.7</td>
<td>27.7 ± 4.4f</td>
</tr>
<tr>
<td>Ouabain-insensitive</td>
<td>16.1 ± 2.4</td>
<td>18.1 ± 5.6</td>
</tr>
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Values are means ± SD. *p<0.05; †p<0.02; ‡p<0.01, compared with values in normotensive subjects.
erythrocyte Na and ouabain-sensitive \(^{86}\)Rb uptake in hypertensive and normotensive subjects was \(-0.40 \) (\(p<0.1\)) and 0.01, respectively. In hypertensive subjects, there was no relationship between renin status (PRA) and erythrocyte Na, \(r = -0.03\), \(n=19\), or between renal function (creatinine clearance) and ouabain-sensitive \(^{86}\)Rb uptake by erythrocytes \(r = 0.22\), \(n=16\).

In a separate group of subjects whose clinical characteristics (not shown) were similar to those of subjects represented in Table 1, erythrocyte Na/K, was correlated with the total \(^{3}H\)ouabain-binding sites per erythrocyte (Figure 2). The correlation coefficient \(r\) between erythrocyte Na/K, and ouabain-binding sites in normotensive and hypertensive subjects was \(-0.53\), (NS) and \(-0.85 \) \((p<0.01)\), respectively. Erythrocyte Na/K, in these normotensive and hypertensive subjects was \(0.117 \pm 0.009\) and \(0.104 \pm 0.012\), \((p<0.02)\). The difference in the total number of ouabain-binding sites per erythrocyte in the two groups, \(720 \pm 64\) versus \(801 \pm 140\), did not reach statistical significance \((p>0.10)\).

**Discussion**

Our contribution to the study of erythrocyte Na transport in hypertension has been the application of techniques that permit measurements to be taken within seconds (in the case of cation concentrations\(^{17}\)) or within minutes (in the case of K transport) after the withdrawal of blood. The erythrocytes are studied in the subjects' own plasma. Our techniques preclude the separation of white blood cells and platelets from the erythrocytes, but we have shown that their presence in the cell pellets has no detectable effect on erythrocyte cation measurements.\(^{17}\) Per liter of erythrocytes, our measurements of erythrocyte Na and K content are 12.9 and 108 mmol, respectively. These measurements are in agreement with published results of other investigators studying unwashed erythrocytes.\(^{21}\)

The presence of white blood cells and platelets does not seem to affect the measurements of erythrocyte K transport either, as outlined in the present study. Using \(^{86}\)Rb uptake as an analogue for K transport, we found that our measurements of ouabain-sensitive and ouabain-insensitive K transport, although somewhat high, were in general agreement with published results.\(^{22,23}\) (The comparison required the conversion of published results, expressed per liter of erythrocytes, to units per liter of cell water.) Measurements of K transport in the subjects' own plasma may account for the high values, as it has been shown that serum or plasma stimulates pump activity by increasing Na permeability.\(^{16}\) Our techniques for measuring K transport do not require preloading of cells, which has often led to permeability changes as manifested by hemolysis.\(^{10}\) We avoid washing the cells in artificial salt solutions, which, in addition to bringing about rapid changes in transmembrane cation gradients, may remove variable portions of cell Na. While producing some improvements, our methodology also has its pitfalls. In the present study, the values for erythrocyte Na, are about 11% lower than in a previous study,\(^{17}\) while K, values are the same. We suspect that the shift in the Na values was due to Na contamination of reagents or diluents or both. This finding points out the need for the study of parallel controls in this type of experimentation. The use of historical controls must be avoided.

Despite the shift of erythrocyte Na values in the present study, we confirmed our previous findings of a reduction of erythrocyte Na, and Na/K, in hypertensive subjects.\(^{17}\) Erythrocyte Na, in hypertensive subjects was unrelated to 1-hour ambulatory PRA, but our sample included few subjects with low or high renin hypertension. The relationship between erythrocyte Na gradients and renin status in hypertensive subjects will have to be investigated further in a much larger sample of subjects.

The new finding of our study is the demonstration of increased ouabain-sensitive K transport of erythrocyte Na.
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cytes of hypertensive subjects, indicating increased Na\(^+\)-K\(^+\) pump activity. The distribution of data points for ouabain-sensitive \(^{86}\)Rb uptake of erythrocytes in normotensive and hypertensive subjects (see Figure 1) is similar to the previously published distribution of erythrocyte Na\(^+\) in these two groups.\(^{17}\) Erythrocyte Na\(^+\) and ouabain-sensitive \(^{86}\)Rb uptake in normotensive subjects are evenly distributed over a wide range, whereas in hypertensive subjects, erythrocyte Na\(^+\) values aggregate around the lower range and ouabain-sensitive \(^{86}\)Rb uptake around the upper range of values in controls. These findings suggest that the reduction in erythrocyte Na\(^+\) in hypertensive subjects may be functionally related to the increased pump activity. In the present study, the correlation between erythrocyte Na\(^+\) and pump activity in the same hypertensive subjects was of borderline statistical significance, but the two measurements were obtained on different days, usually 2 or more weeks apart. Additional support for a cause and effect relationship between reduced Na\(^+\) and increased pump activity of erythrocytes in hypertensive subjects was derived from a separate group of hypertensive subjects whose erythrocyte Na\(^+\)/K\(^+\) was inversely related to the number of ouabain-binding sites per erythrocyte. In these experiments, our estimates of ouabain-binding sites were higher than those reported by others but lower than the theoretical estimate of 1000 binding sites per erythrocyte.\(^{1, 4, 24}\) The measured number of binding sites reported by Glynn\(^{24}\) was between 600 and 700 per erythrocyte, which agrees with our estimate. In patients receiving prednisone, the number of binding sites is greater than 800.\(^{23}\) Finally, erythrocyte Na\(^+\)-K\(^+\) pump activity in our hypertensive subjects was unrelated to their renal function, but our sample did not include patients with clinically significant renal insufficiency.

Other investigators have also reported increased erythrocyte Na\(^+\)-K\(^+\) pump activity in human hypertension.\(^{2, 3}\) Garay and co-workers\(^2\) measured Na and K transport in erythrocytes that were Na-loaded and K-depleted by incubation in p-chloromercuribenzenesulphonate (PCMBS). These data have been criticized because of the unphysiological conditions of the experiments.\(^{10}\) PCMBS, a sulfhydryl reagent, may cause an irreversible increase in membrane permeability and may attack other essential sulfhydryl groups in the cell membrane besides those of Na\(^+\), K\(^+\)-ATPase. Woods and co-workers\(^3\) used \(^{86}\)Rb uptake as a measure of RBC Na\(^+\)-K\(^+\) pump activity, which does not require pre-loading of cells. They found that the activity of the ouabain-sensitive Na\(^+\)-K\(^+\) pump was greater in white hypertensive subjects than in normotensive controls.\(^3\) The experiments were conducted in a K-free solution, and the results, expressed in terms of \(^{86}\)Rb uptake, showed a low level of transport. Others, using the same methods, found no difference between the erythrocyte Na\(^+\)-K\(^+\) pump activity of hypertensive and normotensive subjects.\(^4, 5\)

Assuming that erythrocytes are representative of nucleated cells in general, the findings of the present study are in agreement with the evidence for increased cellular ionic turnover in a variety of experimental models of hypertension, particularly mineralocorticoid-induced hypertension.\(^{25, 26}\) It is now generally accepted that aldosterone, the model mineralocorticoid, stimulates Na transport and lowers steady state intracellular Na\(^+\) in a variety of tissues.\(^{27}\) The stimulation of Na transport is due in part to the synthesis of new aldosterone-induced protein, which includes the transport Na\(^+\), K\(^+\)-ATPase itself, and in part to an apparent increase in the activity of the enzyme.\(^{28}\) A reduction in steady-state Na\(^+\) was also found when fibroblasts or vascular smooth muscle cells were cultured in the serum of experimental animals with low renin renal hypertension, suggesting that aldosterone-like compounds may play a role in the pathogenesis of this type of hypertension.\(^{29, 30}\) The cellular effects of aldosterone may also be seen with the use of pharmacological doses of glucocorticoids, perhaps due to occupancy of mineralocorticoid receptors.\(^3\) In prednisone-treated patients, Kaji and co-workers\(^2\) found reduced erythrocyte Na\(^+\), increased erythrocyte K transport, and an increased number of \(^{3}H\)ouabain-binding sites per erythrocyte. They reported a lag period of 7 to 8 weeks between the initiation of prednisone administration and the reduction in erythrocyte Na\(^+\), suggesting that the observed changes in erythrocyte Na transport occurred during erythropoiesis. The findings of Kaji et al.\(^{23}\) in prednisone-treated subjects are similar to our findings in hypertensive subjects. The similarities suggest a common pathogenesis. Aldosteronelike mineralocorticoids have been suspected to play a role in some forms of human hypertension.\(^{31}\) It remains to be seen whether the causative agent in human hypertension is a mineralocorticoid or an unrelated compound with steroidlike activity.

In summary, we have confirmed our previous finding of reduced erythrocyte Na\(^+\) and Na\(^+\)/K\(^+\) in hypertensive subjects and were able to relate these changes to an increased erythrocyte Na\(^+\)-K\(^+\) pump activity. The abnormalities of erythrocyte cation fluxes in hypertensive subjects are similar to those induced by aldosterone in vascular smooth muscle cells and by glucocorticoid administration in the erythrocytes of human subjects, suggesting similarities in pathogenesis.

Acknowledgment

The authors are grateful to Dr. Khalil Ahmed, who has provided a detailed protocol for the measurement of ouabain-binding sites per erythrocyte.\(^{20}\)

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Reduced sodium concentration and increased sodium-potassium pump activity of erythrocytes in human hypertension.

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Hypertension. 1987;9:III13
doi: 10.1161/01.HYP.9.6_Pt_2.III13

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