Erythrocyte Membrane Transport in Hypertensive Humans and Rats
Effect of Sodium Depletion and Excess

PETER U. FEIG, PETER P. MITCHELL, AND JOHN W. BOYLAN

SUMMARY Sodium transport by erythrocyte membranes was studied in hypertensive and normotensive humans and in spontaneously hypertensive rats (SHR) and normotensive Wistar-Kyoto rats (WKY). The rate constants of sodium efflux were increased in both hypertensive humans and rats, and this increase was due mostly to an increase in the ouabain-resistant component of efflux. Both the furosemide-sensitive and furosemide-resistant components of efflux were increased. The ouabain-sensitive efflux was also increased, as confirmed by the ouabain-sensitive rubidium influx in rats. In rats, the intracellular sodium content was also increased in the SHR with respect to the WKY. The transport abnormalities of red cell membrane associated with hypertension were similar in humans and rats. In rats, sodium depletion failed to affect the transport abnormality, while sodium load made the difference in transport between SHR and WKY undetectable. Cross-incubation experiments, using plasma and erythrocytes of WKY and SHR, are more suggestive of a flux abnormality that is intrinsic to the cell membrane than of one that is humoral in nature.

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KEY WORDS • sodium-potassium pump • sodium-potassium cotransport • sodium leak • spontaneously hypertensive rats • intracellular cation content

MORE than 2 decades ago, Losse et al.1 described an increase in sodium and water content of red blood cells of patients with essential hypertension compared with normotensive subjects or patients with hypertension of renal origin. Since then, the same groups2-4 and others,5-24 in a rapidly expanding body of literature, have shown alterations in sodium flux across red cell membranes in essential hypertension. These abnormalities involve multiple transport systems: the ouabain-sensitive sodium pump or ATPase activity,5, 20, 21, 23, 25, 26 the ouabain-resistant, furosemide-sensitive transport or sodium-potassium cotransport,6, 8, 13, 15, 16 sodium-lithium countertransport,8, 11, 18, 22, 31 and passive sodium leak.3, 17, 19 There is lack of agreement about the magnitude, the direction,9, 27 and even the existence itself20, 23, 28, 29 of these abnormalities, a disagreement that may relate in part to severity of hypertension,15, 16 geography,9, 30, 31 sex,17, 18, 27, 28, 32 and race.24, 27, 31, 33-35

Similar erythrocyte cation transport abnormalities exist in the genetically hypertensive rat compared with its normotensive control.2, 13, 36-43 This study presents evidence of sodium flux abnormalities in red blood cells of humans with essential hypertension as well as similarities of these transport abnormalities in humans and genetically hypertensive rats and attempts to clarify some of the mechanisms of these abnormalities in the rat model of the disease.

Materials and Methods

Human Subjects

The 15 hypertensive subjects had mild to moderate hypertension (diastolic blood pressures > 90 mm Hg on repeated measurements), no evidence on standard workup (urinalysis, blood urea nitrogen, serum electrolyte, and creatinine values) of a secondary cause of hypertension, and a family history for hypertension. They were either never treated for hypertension or treated by thiazide diuretics only, with the diuretic
The animals were fed a standard Purina diet (Ralston-Purina Laboratory Rodent Chow, Ralston-Purina, St. Louis, MO) and ad libitum distilled water except during two experimental protocols. In one of the protocols (between age 6.5 and 8 months), the animals were sodium depleted for 6 weeks by feeding them a sodium-deficient diet (No. 902904, ICN Nutritional Biochemicals, Cleveland, OH) and adding chlorothiazide (10-15 mg/day) to the drinking water. Sodium deprivation, chlorothiazide was discontinued at least 2 weeks before the studies. The 18 normotensive subjects had no family history of hypertension, were not taking any medications, and were selected to be similar to the hypertensives in age, weight, sex, and race.

**Rats**

Spontaneously hypertensive rats (SHR) and the normotensive control strain from which the SHR were derived, the Wistar-Kyoto rats (WKY; Laboratory Supply, Indianapolis, IN) were studied at 5 to 15 months of age. Systolic blood pressure levels were determined by the tail-cuff method (Narco Bio-Systems, Inc., Houston, TX).

The animals were fed a standard Purina diet (Ralston-Purina Laboratory Rodent Chow, Ralston-Purina, St. Louis, MO) and ad libitum distilled water except during two experimental protocols. In one of the protocols (between age 6.5 and 8 months), the animals were sodium depleted for 6 weeks by feeding them a sodium-deficient diet (No. 902904, ICN Nutritional Biochemicals, Cleveland, OH) and adding chlorothiazide (10-15 mg/day) to the drinking water. Sodium deprivation, chlorothiazide was discontinued for 4 days and Na flux studies were performed. In another protocol (between age 9 and 9.5 months), the rats were sodium loaded for 14 days by adding 1% NaCl to the food and replacing distilled drinking water with 0.9% saline solution; Na flux studies were performed while the rats were on the high sodium intake.

**Erythrocyte Studies**

Heparinized blood was obtained from the antecubital vein in humans and from the retroorbital sinus in rats. At 13 to 15 months of age, the rats were killed by aortic puncture and the blood used for the cross-incubation studies. The WKY and SHR were paired throughout the studies, and their blood was always studied in parallel.

The erythrocytes were separated from the plasma and buffy coat and washed three times (2000 g for 5 minutes at 4°C) in a suspending solution containing (mmol/L) 135 NaCl, 5 KCl, 1 MgCl₂, 0.5 CaCl₂, 5 Na₂HPO₄, 1 NaH₂PO₄, and 10.1 dextrose, with an osmolality of 285 to 290 mosm/kg of H₂O and a pH of 7.40. For the cross-incubation studies, erythrocytes from WKY and SHR were then preincubated for 5 hours at 37°C in the animal's own plasma (as control) or in the plasma of its pair SHR and WKY, and subsequently washed as described below.

To measure the rate constant of Na efflux, erythrocytes were incubated in a shaking incubator at approximately 40% hematocrit at 37°C in the suspending solution for 2 to 3 hours with tracer Na. (For the cross-incubation studies, the Na was added during the incubation in plasma.) Subsequently, extracellular Na was removed by washing the erythrocytes three times (by centrifugation at 2000 g for 5 minutes at 4°C and resuspension with the suspending solution at 4°C); the erythrocytes were then resuspended to approximately 4% hematocrit and placed in a shaking incubator at 37°C in flasks containing either ouabain (final concentration 10⁻⁴ for human cells and 10⁻³ M or 10⁻² M for rat cells), both ouabain and furosemide (10⁻³ M final concentration), or no inhibitors (equivalent volume of carrier, the same suspending solution, added for control). Aliquots of cell suspension were obtained at times 0, 20, and 60 minutes for human cells and times 0, 10, 20, and 30 minutes for rat cells) and centrifuged immediately at 10,000 g for 0.5 minute (Microcentrifuge B, Beckman Instruments, Palo Alto, CA), and the supernatant separated. Gamma emission was counted in the supernatant and in the original cell suspension, and the rate constant (k) of ²²Na efflux was determined from the slope of the line relating ln(1-R/R₀) to time, where R represents the counts in the cell at time t (in hours, thus k is given in the units of hr⁻¹) and R₀, the counts in an equal volume of cell suspension. The quantity 1-R/R₀ represents the fraction of radioactivity in the original cell suspension remaining in the cells. This relationship is linear during the assay time, as at the low hematocrit used for efflux, the background is relatively small. Thus, the total (kₒ), ouabain-resistant (kₒᵤ), ouabain-sensitive (kₛ, equal to kₒ - kₒᵤ), ouabain-resistant and furosemide-resistant (kₒₐ), and furosemide-sensitive (kₛ, equal to kₒ - kₒₐ) components of the rate constant of sodium efflux were determined. To measure the rate constant of ⁸⁶Rb influx in rats, the suspending solution was modified only by replacing the 5 mM K with 4 mM K and 1 mM Rb. A tracer amount of ⁸⁶Rb was added to erythrocyte suspensions of approximately 5 to 8% hematocrit, at 37°C in a shaking incubator, with and without 10⁻² M ouabain, as previously described. Aliquots of suspension were obtained at times 0, 30, and 60 minutes and centrifuged immediately (10,000 g for 0.5 minute) through silicone fluid (Versilub F50, General Electric, Waterford, NY), and the red cells were separated. Gamma emission was counted in the cells and in the cell suspension, and the rate constant was determined from the slope of the line relating ln(1-R/R₀) to time where R represents counts in the cells at time t and R₀, counts in the cells at infinite time. Rₛ is calculated from steady state equilibrium as Rₛ (counts in the suspending solution) divided by 1 + {[T (Kₛ)/(Kₛ + Hct)]} where the extracellular potassium concentration (Kₛ) was taken as 5 mM (since the suspending solution contained 4 mM K plus 1 mM Rb), intracellular potassium concentration (Kₛ), as 107 mM (from our measurements), and hematocrit measured in triplicate samples (10 minute centrifugation, model MB microcapillary centrifuge, International Equipment Company, Boston, MA).

The intracellular cation content was measured on the erythrocytes incubated at 40% hematocrit at 37°C in suspending solution. After triplicate hematocrit determinations and two repeated resuspensions in 200-fold volume of 100 mM MgCl₂ and 10 mM Tris buffer (270 mosm/kg of H₂O, pH = 7.4) at 4°C and centrifugations at 4°C, the cells were lysed with 40-fold volume.
of double distilled water, the lysate was centrifuged and potassium and sodium concentrations were measured by flame photometry against a lithium standard (IL 443, Instrumentations Laboratory, Waltham, MA).

Data are given as mean ± SEM. For humans, the data were analyzed by unpaired two-tailed Student's t test. Because in some human subjects multiple measurements at different days were obtained, the results for each individual were averaged and used as a single value for that subject. The results for the rats were analyzed either by paired two-tailed Student's t test (for studies on a single occasion) or by multivariate analysis of variance (when multiple occasions were used and compared).

<table>
<thead>
<tr>
<th>TABLE 1. Age, Weight, Sex, Race, and Blood Pressure of Hypertensive and Matched Normotensive Humans</th>
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</thead>
<tbody>
<tr>
<td>Age (yr)</td>
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<tr>
<td>----------</td>
</tr>
<tr>
<td>Normotensive</td>
</tr>
<tr>
<td>Hypertensive</td>
</tr>
<tr>
<td>p*</td>
</tr>
</tbody>
</table>

Values are means ± SEM. *p by unpaired two-tailed Student's t test.

Results

Table 1 compares data for age, weight, sex, race, and blood pressure of the hypertensive subjects and normotensive controls. The higher weight of the hypertensive group is due mostly to the presence of three markedly obese hypertensive subjects and only one equally obese normotensive one. As there is no a priori reason for removal of these four subjects, they are included. (Their removal from the series makes the weights in the two groups almost identical and increases the differences between the two groups in the sodium transport parameters described below.)

Rate constants of sodium efflux are graphically represented in Figure 1. The rate constant of uninhibited Na efflux was 0.409 ± 0.016 hr⁻¹ in normal subjects and 0.501 ± 0.034 hr⁻¹ in essential hypertensive subjects (p = 0.015). The ouabain-resistant component of efflux was 0.121 ± 0.008 hr⁻¹ in normal subjects and 0.160 ± 0.014 hr⁻¹ in essential hypertensive subjects (p = 0.018), while the ouabain-sensitive component was 0.292 ± 0.011 hr⁻¹ in normal subjects versus 0.345 ± 0.025 hr⁻¹ in hypertensive subjects (p = 0.053). Within the ouabain-resistant component, both the furosemide-resistant and the furosemide-sensitive fractions were increased in hypertensive subjects.

Ouabain-resistant and furosemide-resistant efflux was 0.062 ± 0.005 hr⁻¹ in normal subjects versus 0.082 ± 0.008 in hypertensive subjects (p = 0.045); the furosemide-sensitive fraction was 0.052 ± 0.005 hr⁻¹ in normal subjects versus 0.075 ± 0.009 hr⁻¹ in hypertensive subjects (p = 0.032). For the four black subjects, the total rate constant and its components were lower than for the white subjects (total efflux rate constant 0.298 ± 0.019 hr⁻¹ for blacks versus 0.472 ± 0.019 hr⁻¹ for whites, p < 0.002). In addition, there seemed to be less difference (or no difference) between normotensive and hypertensive blacks in the total efflux (0.288 hr⁻¹ for the two black normotensive subjects versus 0.309 hr⁻¹ for the two black hypertensive subjects) and for the measured components of efflux. While this finding is in line with the racial differences shown by others, the number of blacks in our study is small and precludes major conclusions. Removal of the four black subjects (for which we had no a priori reason) markedly increases the difference and the statistical significance of the difference between normotensive and hypertensive subjects.

Table 2 compares body weight and systolic pressures of WKY and SHR at 4 and 14 months of age and average intracellular sodium and potassium concentrations in red cells measured between 6 months and 15 months of age. Hypertension is well established in this strain of SHR by the tenth week of life. Potassium concentrations in red cells of SHR and WKY were not different, but intracellular sodium concentration was
higher in SHR (7.3 ± 0.3 mEq/L cells) than in WKY (6.2 ± 0.3 mEq/L of cells, p = 0.033). The rate constants of sodium efflux measured in 10 pairs of rats (see Figure 1), while higher than in humans, showed similar differences between the normotensive and hypertensive animals. For uninhibited efflux, the rate constant was 1.513 ± 0.041 hr⁻¹ in WKY and 1.657 ± 0.038 hr⁻¹ in SHR (p = 0.013). The ouabain-resistant component of efflux was 0.688 ± 0.012 hr⁻¹ in WKY and 0.803 ± 0.030 hr⁻¹ in SHR (p = 0.003), while the ouabain-sensitive component was 0.829 ± 0.043 hr⁻¹ in WKY versus 0.856 ± 0.054 hr⁻¹ in SHR (p = 0.606). Within the ouabain-resistant component, as in the human study, the furosemide-resistant efflux was 0.549 ± 0.010 in WKY versus 0.625 ± 0.028 in SHR (p = 0.021) and the furosemide-sensitive efflux was 0.139 ± 0.014 in WKY versus 0.178 ± 0.010 in SHR (paired p = 0.111, unpaired p = 0.039); both significantly increased in SHR.

As seen in Figure 1, the relative inhibition of sodium efflux by ouabain was less in rats than in humans. This phenomenon, related to the well-known resistance of the rat to the effect of ouabain, caused our results of ouabain-sensitive component to be an underestimate, of those of ouabain-resistant and ouabain-and-furosemide-sensitive component to be an overestimate, of differences between strains. Table 3 combines results of five studies on eight pairs of rats between the ages of 5 to 15 months, analyzed by analysis of variance. As seen, total efflux rate constant and its ouabain-insensitive components were significantly increased in SHR and the difference from WKY values was maintained throughout the 10-month period of study.

Sodium depletion, whether for 2 or 6 weeks, had no effect on the differences in total and ouabain-resistant sodium efflux in erythrocytes of the two strains (Table 4). After 2 weeks of sodium loading, however, all differences in fluxes between the two strains disappeared (Table 5).

Data from the cross-incubation studies are displayed in Table 6. As seen, whether preincubated in homologous plasma or cross-incubated, the differences in sodium efflux between WKY and SHR erythrocytes remained. There was no effect of cross-incubation...
TABLE 4. Rate Constants of $^{22}$Na Efflux (in hr$^{-1}$) of 8 Wistar-Kyoto and 8 Spontaneously Hypertensive Rats, Studied Twice After 2 and 6 Weeks of Sodium Depletion

<table>
<thead>
<tr>
<th></th>
<th>Total</th>
<th>Ouabain resistant</th>
<th>Ouabain sensitive</th>
<th>Ouabain and furosemide resistant</th>
<th>Furosemide sensitive</th>
</tr>
</thead>
<tbody>
<tr>
<td>WKY</td>
<td>1.669 ± 0.050</td>
<td>0.809 ± 0.016</td>
<td>0.860 ± 0.045</td>
<td>0.586 ± 0.014</td>
<td>0.223 ± 0.017</td>
</tr>
<tr>
<td>SHR</td>
<td>1.816 ± 0.046</td>
<td>0.923 ± 0.035</td>
<td>0.894 ± 0.058</td>
<td>0.669 ± 0.033</td>
<td>0.272 ± 0.032</td>
</tr>
<tr>
<td>p*</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>0.491</td>
<td>0.004</td>
<td>0.108</td>
</tr>
</tbody>
</table>

Values are averaged (mean ± SEM).
SHR = spontaneously hypertensive rats; WKY = Wistar-Kyoto rats.
*p by multivariate analysis of variance.

TABLE 5. Rate Constants of $^{22}$Na Efflux (in hr$^{-1}$) of 8 Wistar-Kyoto and 8 Spontaneously Hypertensive Rats, Studied After 2 Weeks of Sodium Loading

<table>
<thead>
<tr>
<th></th>
<th>Total</th>
<th>Ouabain resistant</th>
<th>Ouabain sensitive</th>
<th>Ouabain and furosemide sensitive</th>
<th>Furosemide sensitive</th>
</tr>
</thead>
<tbody>
<tr>
<td>WKY</td>
<td>1.653 ± 0.040</td>
<td>0.863 ± 0.039</td>
<td>0.790 ± 0.051</td>
<td>0.590 ± 0.021</td>
<td>0.273 ± 0.022</td>
</tr>
<tr>
<td>SHR</td>
<td>1.603 ± 0.057</td>
<td>0.866 ± 0.028</td>
<td>0.737 ± 0.048</td>
<td>0.591 ± 0.017</td>
<td>0.276 ± 0.023</td>
</tr>
<tr>
<td>p*</td>
<td>0.489</td>
<td>0.941</td>
<td>0.460</td>
<td>0.971</td>
<td>0.935</td>
</tr>
</tbody>
</table>

Values are means ± SEM.
SHR = spontaneously hypertensive rats; WKY = Wistar-Kyoto rats.
*p by multivariate analysis of variance.

TABLE 6. Rate Constants of $^{22}$Na Efflux (in hr$^{-1}$) of 5 Wistar-Kyoto and 5 Spontaneously Hypertensive Rats, Studied After Preincubation in Homologous Plasma and After Cross-incubation

<table>
<thead>
<tr>
<th></th>
<th>Homologous preincubation</th>
<th>Cross-incubation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total</td>
<td>Ouabain resistant</td>
</tr>
<tr>
<td>WKY</td>
<td>1.420 ± 0.052</td>
<td>0.720 ± 0.038</td>
</tr>
<tr>
<td>SHR</td>
<td>1.612 ± 0.083</td>
<td>0.816 ± 0.038</td>
</tr>
<tr>
<td>p*</td>
<td>0.011</td>
<td>0.040</td>
</tr>
</tbody>
</table>

Values are means ± SEM.
SHR = spontaneously hypertensive rats; WKY = Wistar-Kyoto rats.
*p by unpaired two-tailed Student's t test.

(right-hand columns compared with left-hand columns) on membrane fluxes of cells from either WKY or SHR.

**Discussion**

Similar abnormalities of sodium transport by red cell membranes are found in humans with mild to moderate essential hypertension and in rats with spontaneous hypertension. When the cells are incubated in a medium that mimics the physiological state (in terms of temperature, osmolarity, pH, and potassium concentration), this abnormality consists of an increase in the rate constant of total sodium efflux. Others have reported a similar increase in total sodium efflux by red cells of hypertensive humans3, 21 and rats36, 40 and results of the present study substantially agree with these investigators that the greatest difference is found in the ouabain-resistant fraction.

If the sodium efflux that is measured in the presence of both ouabain and furosemide is defined as leak, then the increase of this component in red cells of hypertensive humans (see Figure 1) and rats (see Figure 1; Tables 3 and 4) supports published findings of others in studies of human1, 17, 21 and animal19, 42, 43 hypertension. An increase in the leak component of sodium flux would account for the greater intracellular sodium content of red cells from hypertensive subjects (see Table 2), noted also by others for both erythrocytes31, 40 and leukocytes44. It is apparent that the sodium-sodium exchange (or sodium-lithium counter-transport), which is also resistant to both ouabain and furosemide and has been shown to be increased in essential hypertension3, 19, 21, could not cause the observed increase in intracellular sodium content as it does not cause net transport of this ion.

An increase in the furosemide-sensitive component of efflux, presumed to measure sodium-potassium cotransport, has been described by other investigators in
humans" and rats. For reasons that are presently unknown, sodium-potassium cotransport measured in European hypertensive subjects was found to be decreased. It is apparent that other factors, such as geography and ethnicity, may play a role in this phenomenon.

Previous studies showing a decreased activity of the ouabain-sensitive sodium pump in red cells of essential hypertensive subjects have been interpreted as evidence for the presence of a sodium transport inhibiting factor in the plasma of these patients. We were unable to confirm a decrease in this active transport component of sodium efflux. In fact, the data suggest a slight increase in efflux in both hypertensive humans (see Figure 1; \( p = 0.053 \)) and rats (see Table 3; \( p = 0.071 \)). This finding was confirmed in a separate group of rats by measuring ouabain-sensitive \(^{86}\)Rb influx (\( p = 0.006 \)), which is in line with other reports and could be secondary to increased intracellular sodium concentration.

Evidence from renal homografts in rats and humans supports a primary role of the kidney as a determinant of hypertension. A major present hypothesis presupposes a genetic defect in the kidney that results in a diminished ability to excrete sodium. Sodium transport abnormalities and hypertension are then thought to be due to humoral factors released in response to sodium retention. If this were the case, then the sodium transport abnormalities should be ameliorated by sodium depletion and perhaps exaggerated by sodium loading. In fact, neither result was found. The transport differences in red cells of SHR and WKY persisted following sodium depletion (see Table 4) and actually disappeared after 2 weeks of sodium loading (see Table 5). The reason for this disappearance of the difference between WKY and SHR is unclear. One explanation, that the SHR has its membrane defect already maximally induced because of sodium excess while the defect is induced in the WKY only after a sodium load, could in fact support a hormonal cause for the membrane defect. This question deserves further investigation.

Finally, in our cross-incubation studies, as in those of others, the red cell membrane transport characteristics were not transferred to WKY cells by the plasma of SHR, nor did the plasma of WKY normalize the red cell membranes of SHR (see Table 6). Although the direct in vitro effect of plasma on membrane flux was not tested, our results suggest that the observed abnormalities are intrinsic to the cell membrane rather than secondary to a hormonal factor. Pston et al. explained this failure to support a hormonal cause for the red cell membrane abnormality by the small volume of plasma per number of red cells in the incubation medium, as they were in fact successful in transferring the hypertensive mode of transport to human leukocytes, using fewer cells incubated in plasma of hypertensive subjects. In the latter study, however, a decrease in the ouabain-sensitive sodium efflux had been found in red cells, as well as in leukocytes of hypertensive humans.

Although resolution of the differences between the diverse studies in the literature awaits both methodological standardization and better understanding of the transport systems being measured, our findings clearly indicate that a membrane transport abnormality is present in genetic hypertension. In addition, the fact that we found these abnormalities to be similar in humans and rats with genetic hypertension further validates the rat model for studies of these cell membrane abnormalities and of their pathophysiological role in genetic hypertension.

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