Race, Sex, and Family History of Hypertension and Erythrocyte Sodium Pump \[^3\text{H}\]Ouabain Binding

Emel Songu-Mize, Bruce S. Alpert, and Elaine S. Willey

We studied the binding properties of \[^3\text{H}\]ouabain to erythrocytes from normotensive children (n=83) between the ages of 10 and 18 years (mean resting arterial pressure: 102/57 mm Hg) from normotensive and essential hypertensive parents. Arterial blood pressures of 101/57 and 104/57 mm Hg (subjects with normotensive and hypertensive parents, respectively) were not significantly different between the two groups. Forty-four children had normotensive parents and 39 had hypertensive parents, 51 were white and 32 were black, and 41 were girls and 42 were boys. By using the \[^3\text{H}\]ouabain-binding technique, we determined the density of sodium pump sites and the equilibrium dissociation constants in erythrocytes from these children. Possible effects of race, sex, or parental hypertension status on pump sites and dissociation constants were tested with a three-way analysis of variance (ANOVA). Race had a major effect on the dissociation constant: blacks had a significantly higher value than did whites (p=0.002). We also found a race by sex by parental hypertension status interaction (p=0.04) with black girls with hypertensive parents having the highest value. There was no effect of race, sex, or status on sodium pump site density. Age, height, weight, resting arterial blood pressure, and plasma Na\(^+\) and K\(^+\) concentrations did not correlate with the dissociation constants. These data suggest that, among the groups we studied, black girls with hypertensive parents had erythrocytes with the lowest binding affinity for ouabain. In addition, race had a strong effect on the binding affinity of ouabain to human erythrocytes, with blacks having lower affinities than whites. Because the black population has a much higher prevalence of hypertension than the white population and black women have the highest prevalence of hypertension, this decrease in the affinity of ouabain-binding to the erythrocyte sodium pump molecule may be a possible predictor of adult hypertension. (Hypertension 1990;15:146-151)

Several abnormalities of cation transport in erythrocytes and leukocytes of patients with essential hypertension have been reported.\(^1,2\) Alterations in furosemide-sensitive and ouabain-sensitive sodium efflux\(^3-6\) and sodium-lithium countertransport rates\(^7-10\) and the concentrations of intracellular sodium have been found in patients with essential hypertension.\(^11,12\) Abnormalities in sodium transport pathways in other tissues, such as the vasculature, have also been noted in some animal models of hypertension.\(^13-16\) In some reports, normotensive offspring of hypertensive individuals manifested some of these sodium transport abnormalities.\(^6,17,18\) In spite of extensive knowledge related to cation transport in hypertensive persons, findings related to ouabain-sensitive or the sodium pump-driven transport system are less definitive than findings for other transport systems. Moreover, binding properties of \[^3\text{H}\]ouabain to red blood cells in the hypertensive subjects and their offspring and the effect of race and sex have not been studied thoroughly.

In the search for a possible predictor of adult hypertension, we determined whether an abnormality exists in the number of erythrocyte sodium pump sites and in affinity for \[^3\text{H}\]ouabain binding to the sodium pump molecules in normotensive children of hypertensive parents. We also analyzed possible effects of sex and race on erythrocyte sodium pump properties in these children.
Methods

Subjects

We studied 83 healthy children between the ages of 10 and 18 for our erythrocyte [3H]ouabain-binding experiments. Children were recruited from schools, churches, and social organizations to participate in a blood pressure study. After a brief physical examination, children with cardiovascular abnormalities were eliminated from the study. Informed consent was obtained from the child's parent. The protocol was approved by the institutional review board for human subjects research.

Binding Studies

Erythrocyte [3H]ouabain-binding studies used a modification of a procedure described by Ash et al. Blood samples (10 ml) were collected into chilled heparinized tubes at 8:00 AM and centrifuged at 1,300 rpm for 10 minutes in a Beckman refrigerated tabletop centrifuge (Accuprin FR, Beckman Instr., Inc., Fullerton, California). Packed erythrocytes were stored in the refrigerator with 1 ml acid citrate dextrose solution (2.2 g sodium citrate trisodium, 0.8 g citric acid, and 2.45 g anhydrous dextrose in 100 ml double-distilled deionized water) until use.

Binding assays were performed within 48 hours of blood collection. Storage of the erythrocytes for 48 hours in citrate-dextrose buffer did not affect binding properties. This was verified by initial storage time experiments. On the day of the assay, erythrocytes were washed with 140 mM ice-cold choline chloride, three times the volume of cells, by centrifuging for 10 minutes and discarding the supernatant. The wash was repeated three times. Cells were resuspended in a buffer containing 140 mM NaCl, 30 mM HEPES, and 10 mM dextrose (pH 7.4) to give a hematocrit of 15–20%. A 20 µl aliquot was put aside for protein determination. Aliquots (400 µl) of cell suspension in citrate-dextrose buffer did not affect binding properties. This was verified by initial storage time experiments. On the day of the assay, erythrocytes were washed with 140 mM ice-cold choline chloride, three times the volume of cells, by centrifuging for 10 minutes and discarding the supernatant. The wash was repeated three times. Cells were resuspended in a buffer containing 140 mM NaCl, 30 mM HEPES, and 10 mM dextrose (pH 7.4) to give a hematocrit of 15–20%. A 20 µl aliquot was put aside for protein determination. Aliquots (400 µl) of cell suspension were incubated with 0.5, 1, 2.5, 5, 10, 15, 25, and 50 nM [3H]ouabain of specific activity 18–20 Ci/mmol, DuPont, Boston, Massachusetts). For each concentration point, an identical set of tubes containing 1 µM unlabeled ouabain was included to determine nonspecific binding. Final volume was adjusted with buffer to 500 µl.

Cells were incubated for 2.5 hours at a 37°C, gently shaking Dubnoff bath. Binding equilibrium was reached in 2 hours (Figure 1). Reaction was terminated by adding 300 µl dibutyl phthalate (Sigma Chemical Co., St. Louis, Missouri) and centrifuging at high speed for 5 minutes. Supernatant, containing the unbound label, and the phalate separation layer were removed with a Pasteur pipette. Then, 500 µl 5% trichloroacetic acid (TCA) was added to the cells and mixed vigorously with vortex to release the labeled ouabain. The mixture was centrifuged at high speed for 5 minutes and a 400 µl aliquot of the supernatant was counted in 6 ml Biocount (RPI Corp., Mount Prospect, Illinois) scintillation cocktail using a Beckman 7000 liquid scintillation counter.

Specific binding was calculated by subtracting the nonspecific from the total binding. Experiments with [3H]inulin indicated that only 1.102±0.09% of the label introduced to the incubation medium was trapped by the erythrocytes. These experiments included a wide range of counts in the incubation media (10,000–1,000,000 DPMs) of which 0.84 to 1.38% were trapped by the erythrocytes. Binding was expressed as femtomoles of [3H]ouabain bound per milligram erythrocyte protein. The maximum number of binding sites (Bmax) and the dissociation constant (Ka) were estimated by Scatchard analysis of the data. The Scatchard plots were linear for all groups (r²=0.942±0.010, n=83), indicating a single population of specific binding sites for ouabain.

Data Analysis

Data were entered into a VAX 1170 for analyses. Age, height, weight, systolic and diastolic blood pressure, and plasma electrolytes were compared among groups by race, sex, and status using Student's t test. The Statistical Analysis System (SAS) analysis of variance (ANOVA) procedure was used to determine the effects of race, sex, and parental hypertension status and the interactions between these variables. A three-way ANOVA followed by Fisher's least significant difference test was applied. Pearson correlation coefficients were compared within each race, sex, and status to determine the relation between Bmax and age, height, weight, systolic and diastolic blood pressure, and plasma electrolytes. Because none of these variables correlated with Bmax, there was no need to adjust for these variables with an analysis of covariance.

Subject Characteristics

Of the 83 subjects, 32 were black (B) and 51 white (W). 41 were girls (F) and 42 were boys (M). Thirty-nine children with at least one hypertensive parent (BP>160/95 [n=32] or borderline hypertensive, 160/95>BP>140/90 [n=7]) were classified as "children with hypertensive parent" (HT), and 44 children with normotensive parents were classified as "children with normotensive parent" (NT). The parental hypertension status was verified by reports from the parent's doctor. Each subgroup had the following number of subjects: WM-NT, 16; WM-HT, 10; WF-NT, 13; WF-HT, 12; BM-NT, 6; BM-HT, 10; BF-HT, 9; BF-HT, 7.

Casual Measurements

Age, weight, height, resting arterial blood pressure, and plasma Na+ and K+ concentrations are presented in Table 1. Blacks as a group were heavier than whites (p=0.0503). Boys were significantly taller than the girls (p=0.0027), and systolic blood pressure of boys was higher than girls (p=0.0058). There was no difference in age, diastolic blood pressure, and plasma electrolytes among the groups.
TABLE 1. Age, Height, Weight, Resting Arterial Blood Pressure, and Plasma Na⁺ and K⁺ Values

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Age (yr)</th>
<th>Height (m)</th>
<th>Weight (kg)</th>
<th>SBP (mm Hg)</th>
<th>DBP (mm Hg)</th>
<th>Plasma Na⁺ (meq/l)</th>
<th>Plasma K⁺ (meq/l)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>All</td>
<td>13.4±0.3</td>
<td>1.60±0.02</td>
<td>56±2</td>
<td>103±1</td>
<td>55±1</td>
<td>139±0.4</td>
<td>4.28±0.05</td>
<td>83</td>
</tr>
<tr>
<td>White</td>
<td>12.9±0.4</td>
<td>1.58±0.02</td>
<td>53±2*</td>
<td>102±2</td>
<td>54±1</td>
<td>140±0.5</td>
<td>4.38±0.07</td>
<td>51</td>
</tr>
<tr>
<td>Black</td>
<td>14.0±0.5</td>
<td>1.62±0.02</td>
<td>61±3*</td>
<td>105±2</td>
<td>56±2</td>
<td>140±0.8</td>
<td>4.14±0.07</td>
<td>32</td>
</tr>
<tr>
<td>Male</td>
<td>13.3±0.4</td>
<td>1.63±0.02*</td>
<td>59±3</td>
<td>107±2*</td>
<td>53±2</td>
<td>139±0.5</td>
<td>4.32±0.06</td>
<td>42</td>
</tr>
<tr>
<td>Female</td>
<td>13.4±0.4</td>
<td>1.56±0.02*</td>
<td>54±3</td>
<td>99±1*</td>
<td>57±2</td>
<td>139±0.8</td>
<td>4.25±0.09</td>
<td>41</td>
</tr>
<tr>
<td>Normotensive parent</td>
<td>13.1±0.4</td>
<td>1.59±0.02</td>
<td>55±3</td>
<td>103±2</td>
<td>54±2</td>
<td>139±0.5</td>
<td>4.32±0.08</td>
<td>44</td>
</tr>
<tr>
<td>Hypertensive parent</td>
<td>13.7±0.4</td>
<td>1.61±0.02</td>
<td>58±3</td>
<td>103±2</td>
<td>56±2</td>
<td>139±0.8</td>
<td>4.23±0.06</td>
<td>39</td>
</tr>
</tbody>
</table>

Data are presented as mean±SEM. SBP, systolic blood pressure; DBP, diastolic blood pressure.

*Indicate a significant difference between the groups, p<0.05 (see actual p values in text), Student's t test.

Verification of Binding Methods

Time course of [³H]ouabain binding to erythrocytes. The time course of total, nonspecific, and specific [³H]ouabain binding to human erythrocytes is shown in Figure 1. At 37°C, equilibrium for specific [³H]ouabain binding was reached in 2 hours. Therefore, we selected 2.5 hours for our equilibrium binding experiments. The nonspecific binding reached maximum before 5 minutes and was constant for 3 hours. The final ouabain concentration was 5 nM.

Saturability of [³H]ouabain binding to erythrocytes. Specific [³H]ouabain binding to human erythrocytes was saturable; the amount of bound ouabain increased with increasing concentrations of ouabain and reached a plateau. Figure 2 represents a plot of binding saturation curve (Figure 2A) and Scatchard transformation of the data from subject 209 (a 10-year-old black girl with normotensive parents) (Figure 2B). The nonspecific binding was unsaturable as expected. The Scatchard transformation of the data yielded a straight line with a correlation coefficient of 0.958. The mean correlation coefficient for 83 subjects was 0.942±0.010. The KD and the Bmax values were derived from the Scatchard plot; the negative reciprocal of the slope represents the KD and the x intercept represents the Bmax.

Binding Characteristics of [³H]Ouabain to Erythrocytes

The KD and the Bmax variables were evaluated with three-way ANOVA followed by Fisher's least significant difference test to determine if there were race,
TABLE 2. \([3H]\)Ouabain Binding to Human Erythrocytes

<table>
<thead>
<tr>
<th>Subjects</th>
<th>(K_d) (nM)</th>
<th>(B_{max}) (fmol/mg protein)</th>
<th>(n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>All</td>
<td>6.51±0.56</td>
<td>11.02±0.69</td>
<td>83</td>
</tr>
<tr>
<td>White</td>
<td>5.28±0.68*</td>
<td>11.60±0.92</td>
<td>51</td>
</tr>
<tr>
<td>Black</td>
<td>8.79±0.87*</td>
<td>10.24±1.17</td>
<td>32</td>
</tr>
<tr>
<td>Male</td>
<td>6.89±0.79</td>
<td>10.87±1.06</td>
<td>42</td>
</tr>
<tr>
<td>Female</td>
<td>7.18±0.77</td>
<td>10.97±1.04</td>
<td>41</td>
</tr>
<tr>
<td>Normotensive parent</td>
<td>6.55±0.77</td>
<td>10.70±1.05</td>
<td>44</td>
</tr>
<tr>
<td>Hypertensive parent</td>
<td>7.53±0.78</td>
<td>11.15±1.06</td>
<td>39</td>
</tr>
</tbody>
</table>

Values are least-square mean±SEM. Data are analyzed by three-way analysis of variance followed by Fisher's least significant difference test. \(K_d\), dissociation constant; \(B_{max}\), maximum number of binding sites.

*Indicate a significant difference between black and white groups, \(p=0.002\).

sex, or parental hypertension status differences (Table 2). The \(B_{max}\) was 11.02±0.69 fmol/mg protein (LSM±SEM, \(n=83\)). Race, sex, and status had no effect on \(B_{max}\) (Table 2). We found that race had a major effect (\(p=0.002\)) on \(K_d\) with blacks having a significantly higher value (LSM±SEM=8.79±0.87 nM) than did whites (LSM±SEM=5.28±0.68 nM) (Table 2). We also found a significant race by sex by parental hypertension status interaction (\(p=0.04\)) with black girls with a hypertensive parent having the highest value for \(K_d\) (LSM±SEM=12.22±1.81) (Figure 3). Erythrocyte \(K_d\) for \([3H]\)ouabain binding in black girls with a hypertensive parent was significantly higher than that of white boys with hypertensive parents, \(p=0.0058\) and normotensive (\(p=0.0017\)) parents, white girls with hypertensive (\(p=0.0013\)) and normotensive (\(p=0.0061\)) parents, and black girls with normotensive parents (\(p=0.0124\)) (Figure 3).

Correlations

Pearson correlation coefficients were compared within each race, sex, and status to determine the relations between \(K_d\) and age, height, weight, systolic and diastolic blood pressure, and plasma electrolytes. There was no correlation between the \(K_d\) values and any of these variables. Therefore, we did not adjust for these variables with an analysis of covariance.

Discussion

Cation transport abnormalities are known to occur in hypertension.\(^1,2\) Although there is no doubt about the existence of cellular cation transport abnormalities in hypertension, there is still debate about the transport pathways affected and the direction of changes. Elevated erythrocyte sodium-lithium countertransport has been the most consistent finding in human essential hypertension.\(^7,10\) In contrast, the furosemide-sensitive sodium and potassium cotransport rate appears to be reduced in erythrocytes and leucocytes of patients with essential hypertension.\(^3,4,11\) In addition, a high proportion of their children shows this rate reduction as well.\(^5,17,18\) Meyer et al.\(^22\) proposed that a sodium and potassium cotransport pathway defect was inherited and a single gene mechanism was involved.

The findings related to the ouabain-sensitive sodium pump in erythrocytes are less consistent and are mainly concerned with the functional aspects of the sodium pump. Both inhibition and stimulation of the sodium pump, or sodium-potassium adenosine triphosphatase (Na\(^+\),K\(^+\)-ATPase), have been reported. For example, Woods et al.\(^6\) using the \(^86\)Rb uptake method, measured an increased erythrocyte sodium pump activity, both in essential hypertensive patients and in a significant proportion of their normotensive relatives. However, direct measurements of the erythrocyte enzyme Na\(^+\),K\(^+\)-ATPase activity in hypertensive subjects\(^12\) and their normotensive children\(^23\) revealed lower activities than in corresponding controls. A circulating sodium pump inhibitor, shown in patients with essential hypertension\(^24,25\) may cause reduced sodium pump activity reported in some studies. A cause–effect relation of any of the transport defects and the etiology of hypertension have not been established. We also do not know whether any of these abnormalities are associated with a genetic tendency toward development of hypertension. Because reports of erythrocyte sodium pump activity are inconsistent and are mainly concerned with the functional aspects of the sodium pump, we decided to study the Na\(^+\),K\(^+\)-ATPase or the sodium pump in red blood cells in terms of the maximum number of pump units and the affinity of \([3H]\)ouabain binding to the cells in the normotensive children of essential hypertensive individuals. Our purpose was to determine whether any difference in
sodium pump characteristics between these groups exists that may be a biochemical marker for development of adult hypertension. Additionally, we have focused on effects of race and sex. Our data indicate a strong effect of race on ouabain-binding affinity with a significant three-way interaction of race by parental hypertension by sex, that is, black female children with hypertensive parents had erythrocyte Na\textsuperscript{+},K\textsuperscript{+}-ATPase with the lowest affinity for ouabain. The prevalence of hypertension in the black population is much higher than the white population. Furthermore, black women have the highest prevalence of hypertension.\textsuperscript{26} In a recent report, Smith et al\textsuperscript{27} reported that the black female hypertensive population was distinctive in their values for several of the Na\textsuperscript{+},K\textsuperscript{+}-ATPase pump parameters. This group had higher sodium pump sites per erythrocytes compared with other black groups. In addition, black hypertensive women had lower ouabain-sensitive and ouabain-insensitive sodium efflux than the normotensive black women, whereas other hypertensive subjects had higher values than their normotensive counterparts. This subgroup also displayed the highest salt sensitivity of blood pressure compared with all other hypertensive groups.\textsuperscript{28} Smith et al\textsuperscript{27} did not measure the affinity constant for ouabain binding to erythrocytes. Hypertension is a multifactorial disease, and we do not know if our finding of decreased ouabain affinity of the enzyme is one of the risk factors that predispose these children to essential hypertension in adulthood, but our biochemical data are in excellent agreement with the epidemiological data. This calls for a more extensive epidemiological study that would direct particular attention to the black female subpopulation. It has been suggested that a humoral inhibitor of the Na\textsuperscript{+},K\textsuperscript{+}-ATPase, possibly a “digitalislike” substance, is released in individuals with essential hypertension in animals with certain types of genetic and experimental hypertension. Such a substance inhibits the pump activity in the kidney and other tissues such as the vasculature. Inhibition of this enzyme in the kidney facilitates Na\textsuperscript{+} elimination,\textsuperscript{31} whereas its inhibition in the vasculature reduces to an increase in vascular tone, thus contributing to elevation of blood pressure.\textsuperscript{16} In subjects with erythrocyte enzyme possessing the expected affinity for ouabain, red blood cells may act as a sink for the circulating inhibitor and thus protect the vascular smooth muscle enzyme. In subjects with erythrocyte enzymes possessing lower than expected affinity for ouabain, more of the circulating inhibitor will be available for binding to the arterial smooth muscle enzyme resulting in either increased arterial tone or contributing to existing tone.\textsuperscript{32\textsuperscript{34}}

The catalytic \(\alpha\) subunit of Na\textsuperscript{+},K\textsuperscript{+}-ATPase occurs as three isoforms, \(\alpha 1\), \(\alpha 2\), and \(\alpha 3\).\textsuperscript{35,32\textsuperscript{34}} These isoforms are encoded by a multigene family and are expressed in a tissue-specific and developmentally regulated manner.\textsuperscript{34} \(\alpha 2\) has higher affinity for ouabain compared with \(\alpha 1\). \(\alpha 1\) and \(\alpha 2\) appear to be tissue specific, and they differ from each other with respect to some kinetic properties.\textsuperscript{35} Red blood cell Na\textsuperscript{+},K\textsuperscript{+}-ATPase has not been studied thoroughly in terms of its isozymes. Recently, Herrera et al\textsuperscript{36} provided evidence that hypertension induces gene-specific modulation of Na\textsuperscript{+},K\textsuperscript{+}-ATPase messenger RNA in vascular and cardiac tissues in two experimental rat models. Furthermore, this modulation of the enzyme was isoform specific. It is possible that \(\alpha\) isoform of the enzyme differs by several amino acids in blacks (particularly in the group with “low-affinity” or “high-\(K_d\)” enzyme) compared with whites. Therefore, it would be worthwhile to isolate and study the gene for this enzyme in blacks and whites, particularly in the subset of patients with abnormally high \(K_d\) for ouabain binding. The lipid environment of the erythrocyte membrane may also contribute to the affinity of Na\textsuperscript{+},K\textsuperscript{+}-ATPase for ouabain. It has been proposed that the low sensitivity of rat heart enzyme to digitalis emerges from the absence of a lipid barrier on the membrane that regulates the dissociation of cardiac glycosides from their binding sites on Na\textsuperscript{+},K\textsuperscript{+}-ATPase.\textsuperscript{37}

In summary, our biochemical data suggest that race has a strong effect on the binding affinity of ouabain to human erythrocytes, with blacks having lower affinities than whites. Furthermore, black girls with hypertensive parents have erythrocytes with the lowest binding affinity for ouabain. Because the black population has a much higher prevalence of hypertension than the white population, and black women have the highest prevalence for hypertension,\textsuperscript{26} our findings of low affinity of ouabain binding to the erythrocyte sodium pump molecule in normotensive offspring of these groups may be a predictor of adult hypertension. Further studies are needed to prove the hypothesis that this group is predisposed to adult hypertension. Our biochemical findings call for a more extensive epidemiological study focusing on this population and more investigations of the regulation of the Na\textsuperscript{+},K\textsuperscript{+}-ATPase gene.

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References


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