Erythrocyte Anion Exchanger Activity and Intracellular pH in Essential Hypertension

Amalia Alonso, Arantxa Arrázola, Ana García-Diía, Noemí Esparza, Carlos Gómez-Alamillo, Javier Díez

The present study was designed to examine the activity of the sodium-independent chloride-bicarbonate anion exchanger and the sodium-proton exchanger in erythrocytes of 30 normotensive and 35 hypertensive subjects and its relation to the previously reported decrease in erythrocyte pH. Erythrocyte cytosolic pH was measured by the pH-sensitive fluorescent probe 2′-7′-bis(2-carboxethyl)-5(6)-carboxyfluorescein. The activity of the anion exchanger was determined by acidifying cell pH and measuring the initial rate of the net sodium-independent, 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid-sensitive, bicarbonate influx driven by an outward proton gradient. The activity of the sodium-proton exchanger was determined by acidifying cell pH and measuring the initial rate of the net sodium-dependent proton efflux driven by an outward proton gradient. The activity of the anion exchanger was higher in hypertensive than control individuals (18.863±1081 vs 15.629±897 mmol/L cells per hour, P<.05). The activity of the sodium-proton exchanger was higher in hypertensive than control individuals (301±45 vs 162±23 mmol/L cells per hour, P<.005). Basal erythrocyte pH was lower in hypertensive than control individuals (7.27±0.02 vs 7.33±0.01, mean±SEM, P<.05). With the 100% confidence (lower) limit of the normotensive population as a cutoff point, a subgroup of 11 hypertensive patients had an abnormally low erythrocyte pH (<7.19). Compared with patients with normal erythrocyte pH, patients with diminished pH were characterized by a higher activity of the anion exchanger (17.836±956 vs 20.806±1200 mmol/L cells per hour, P<.05) and similar activity of the sodium-proton exchanger (302±53 vs 296±97 mmol/L cells per hour). Erythrocyte pH was inversely correlated with the activity of the anion exchanger. No correlation was found between erythrocyte pH and the activity of the sodium-proton exchanger. These findings show that an association exists between the hyperactivity of the anion exchanger and the diminution of pH in erythrocytes of a subgroup of hypertensive patients. We therefore propose that an abnormal function of the anion exchanger may be involved in the pathophysiology of the hypertensive process via disregulation of cell pH. (Hypertension. 1993;22:348-356.)

KEY WORDS • hypertension, essential • erythrocytes • ion transport • hydrogen-ion concentration • anion exchange

Recently, interest has grown in the way in which the regulation of cell pH may be altered in hypertension, and it has been proposed that altered cell pH regulation may be responsible for both vasoconstriction and vascular hypertrophy in hypertension. However, studies of cell pH levels and regulation in various tissues of patients with hypertension are contradictory. Compared with values obtained in normotensive individuals, cell pH in hypertensive patients has been found to be normal,3-7 increased,8-11 or decreased.12 These conflicting results may be explained in part because pH regulation may differ in different cell types13 but also in part because methodological variations (ie, the presence or absence of HCO₃⁻ in media) may affect the findings.14

With respect to cell pH regulation in erythrocytes of patients with essential hypertension, the abnormalities described include diminished intracellular pH,12 increased activity of the Na⁺-H⁺ exchanger,15 and increased activity of the Na⁺-dependent Cl⁻-HCO₃⁻ anion exchanger.16 Until now, no relation has been found between diminished pH and these two transport abnormalities in erythrocytes from essential hypertensive patients.

Another mechanism critically involved in intracellular pH regulation has been identified in most cells: the electroneutral Na⁺-independent Cl⁻-HCO₃⁻ anion exchanger.13 Because the inward gradient for Cl⁻ usually exceeds that for HCO₃⁻, this transporter catalyzes the net efflux of HCO₃⁻, therefore acting as a cell-acidifying mechanism.17 Sulfonate stilbenes such as 4,4′-diisothio-cyanato-stilbene-2,2′-disulfonic acid (DIDS) inhibit the activity of the Na⁺-independent Cl⁻-HCO₃⁻ anion exchanger in different cell types.17 Band 3 has been identified as the membrane glycoprotein that catalyzes this anionic exchange in mammalian erythrocytes.18,19 We therefore hypothesized that a relationship might exist between increased activity of the Na⁺-independent Cl⁻-HCO₃⁻ anion exchanger and dimin-
TABLE 1. Clinical Parameters in Normotensive Subjects and Hypertensive Patients

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Normotensive</th>
<th>Hypertensive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex (M/F)</td>
<td>20/10</td>
<td>23/12</td>
</tr>
<tr>
<td>Age (y)</td>
<td>41 (26-64)</td>
<td>45 (21-65)</td>
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<tr>
<td>Family history of HBP</td>
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<td>19</td>
</tr>
<tr>
<td>HBP duration (months)</td>
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<td>66 (1-384)</td>
</tr>
<tr>
<td>SBP (mm Hg)</td>
<td>113 (80-140)</td>
<td>162 (130-200)</td>
</tr>
<tr>
<td>DBP (mm Hg)</td>
<td>69 (60-85)</td>
<td>97 (90-122)</td>
</tr>
<tr>
<td>MAP (mm Hg)</td>
<td>84 (67-103)</td>
<td>125 (197-143)</td>
</tr>
<tr>
<td>Heart rate (bpm)</td>
<td>70 (55-88)</td>
<td>74 (52-96)</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>25 (19-31)</td>
<td>27 (21-37)</td>
</tr>
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</table>

HBP, high blood pressure; SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure; bpm, beats per minute. Data are mean and range or number of subjects.

The biochemical evaluation included measurement of the disease.

Methods

Subjects

The study was approved by the institutional Ethics Committees, and all subjects gave informed consent before inclusion. Thirty-five white hypertensive patients were selected from the Hypertension Unit of the San Millán Hospital (Logroño, Spain) and the Department of Internal Medicine of the Center for Biomedical Research (Pamplona, Spain). These patients were considered to have essential hypertension (no known cause of high blood pressure and no associated disease detected after medical examination). All were on a Na+-free diet, and none had received antihypertensive therapy during the month before the study. Five patients had previously received captopril as treatment. The control group consisted of 30 normotensive subjects. These control subjects were normotensive patients of the outpatient clinics of the San Millán Hospital and the University Clinic. Table 1 shows that the two groups of subjects exhibited a similar age and sex distribution. The frequency of family history of hypertension (defined through a medical report) was similar in normotensive and hypertensive individuals (Table 1).

Medical examination consisted of a complete medical history, physical examination, funduscopic observation, and biochemical and echocardiographic evaluation. The biochemical evaluation included measurement of erythrocyte volume by a Coulter Counter autoanalyzer (Coulter Electronics, Luton, UK).

Preparation of Erythrocytes

Early in the morning, venous blood (5 mL) was drawn from each subject maintained in fasting conditions. Blood was collected in heparinized tubes and centrifuged at 1750g for 10 minutes at 4°C. The plasma anduffy coat were aspirated. The erythrocyte pellet was washed twice with cold washing solution containing (mmol/L) NaCl, 118; HCO₃Na, 20; KCl, 2; MgCl₂, 1; CaCl₂, 1; glucose, 10; and Tris-3-(N-morpholino)propanesulfonic acid (MOPS), 15; pH 7.4, at 37°C. The cells were resuspended in the same solution at a hematocrit of 10%. A portion of this cell suspension was set aside to measure hematocrit. A second portion of the cell suspension was used to measure the basal intracellular pH.

For the analysis of the activities of the Na⁺-H⁺ exchanger and the Na⁺-independent Cl⁻-HCO₃⁻ anion exchanger, driven by an outward H⁺ gradient, intracellular pH was acidified in a third portion of the cell suspension. Acidification of erythrocyte pH was performed as follows: Cells were incubated at 1% hematocrit in an acid-loading solution with 400 μmol/L acetazolamide for 30 minutes in a shaking water bath at 37°C. The acid-loading solution contained (mmol/L) KCl, 150; MgCl₂, 1; CaCl₂, 1; glucose, 10; Tris-2-(N-morpholino)ethanesulfonic acid (MES), 20; and sucrose, 30; pH 6, at 37°C. This solution was made hypertonic (osmolality of 360 mOsm) to avoid cell swelling that occurs in acid medium. The mean increase in erythrocyte volume was not greater than 5% of that of the untreated cells.

Measurement of Intracellular pH

Intracellular pH was measured fluorometrically using the pH-sensitive carboxyfluorescein derivative 2′,7′-bis(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF). Suspensions of fresh and acidified cells were incubated at a hematocrit of 1% in different media with BCECF-AM (5 μmol/L) for 30 minutes at 37°C. The incubation media were the cold washing solution and acid-loading solution for fresh and acidified cells, respectively, mentioned above. After incubation, the cells were washed twice and resuspended in identical media, but which lacked the dye. The dye-loaded cells were kept at 4°C until ready for use.

Fluorescence determinations were carried out at 37°C using a flow cytometer FACScan (Becton Dickinson, Mountain View, Calif) with excitation and emission wavelengths set at 488 and 530 nm, respectively. Intracellular pH was calculated from the BCECF fluorescence signals using the slope and intercept of a calibration line generated for each experiment. The calibration curve was performed using the nigericin technique described by Thomas et al. The calibration solutions contained (mmol/L) KCl, 140; MgCl₂, 1; CaCl₂, 1; glucose, 10; nigericin, 0.010; and Tris-MES, 20, for pH lower than 7.0 and Tris-MOPS, 20, for pH higher than 7.0. The solutions were titrated to an external pH between 6.0 and 7.6 (at least five points) using dilute acid (HCl) or base (Tris base). Over this pH range, the fluorescence increased linearly with pH (r values were, 0.99 or higher). A decrease of the linearity was detected at pH values less than 6.0 and more than 7.6. In control experiments, we found that hemoglobin did not interfere with BCECF fluorescence at the referred conditions.

Measurement of Buffering Power

The estimation of acid and base fluxes from intracellular pH measurements requires a knowledge of the intracellular buffering power. The buffering power was determined as described by Roos and Boron. The resting intracellular pH of acidified and BCECF-loaded erythrocytes (pH 6) was first determined. Then 5 mmol/L NH₄Cl was added to the cell suspension, and the new intracellular pH was recorded immediately.
FIG 1. Plot shows time course of Cl⁻-HCO₃⁻-dependent intracellular pH recovery (ΔpHi) after acid load of erythrocytes. For calculation of initial rate, a straight line was fit to the data as shown in inset. ΔpHmax. Maximal intracellular pH recovery; see "Methods."

(approximately 6 seconds). The buffering power was calculated as the ratio of intracellular NH₄⁺ to intracellular pH, where intracellular NH₄⁺ equals the rise in NH₃ produced by the alkaline pulse, and intracellular pH is the difference between resting intracellular pH and intracellular pH after the NH₄Cl pulse. The concentration of intracellular NH₄⁺ was calculated using a pKₐ for NH₃ at 37°C of 8.89 and assuming that NH₃ is in equilibrium across the cell membrane.²³

For cells incubated in HCO₃⁻ solutions, the buffering power of the H₂CO₃ system was not considered, because it constituted approximately 0.5% of the intrinsic buffering capacity described above.

Measurement of the Activity of the Cl⁻-HCO₃⁻ Anion Exchanger

In erythrocytes, the Na⁺-independent Cl⁻-HCO₃⁻ anion exchanger exchanges physiologically either intracellular HCO₃⁻ by extracellular Cl⁻ (in peripheral capillaries) or intracellular Cl⁻ by extracellular HCO₃⁻ (in alveolar capillaries).¹⁷⁻¹⁹ The rate of exchange appears to be the same in the two modes of operation; thus, the activity of this transporter can be assessed in erythrocytes by measuring either of the two exchanges.¹⁷⁻¹⁸ Therefore, the activity of the anion exchanger was assayed as the initial rate of change in intracellular pH after an acid load.

To test the intracellular pH recovery dependent on the Cl⁻-HCO₃⁻ anion exchanger, we divided an aliquot of acidified cells in half. One half was added to an Na⁺-free medium (A) containing (mmol/L) choline chloride, 118; choline-HCO₃, 20; KCl, 2; MgCl₂, 1; CaCl₂, 1; glucose, 10; Tris-MOPS, 20; ouabain, 0.1; and bumetanide, 0.025, pH 8, at 37°C; the other half was added to a similar medium (B) without HCO₃⁻ (HCO₃⁻ had been replaced by Cl⁻) and with 20 μmol/L DIDS. These were the optimal conditions to promote the maximal activity of the anion exchanger. To minimize changes of the concentration of bicarbonate due to loss of CO₂, we prepared the above media freshly at neutral pH and titrated them to the desired alkaline pH the same day of each experiment. The real concentration of bicarbonate present in the media was measured (ABL 300, Radiometer, Copenhagen, Denmark) immediately before addition of the cells. This bicarbonate concentration was never less than 18 mmol/L. On addition of the cells to the medium, fluorescence recording was immediately started and continued for 4 minutes.

Therefore, DIDS-sensitive intracellular pH recovery in acidified cells was used to evaluate the transport activity of the Cl⁻-HCO₃⁻ anion exchanger. This recovery was analyzed by fitting the intracellular pH vs time record to a single-exponential function (Fig 1). For the calculation of the initial rate of recovery, a straight line was fit to the data (Fig 1), and its value (V) was estimated from the equation:

\[ V = K \cdot \Delta \text{intracellular pH}_{\text{max}} / \log e \cdot t \]

where K is the slope of the linear regression line, Δintracellular pH_max is the maximal difference of intracellular pH recovery between cells incubated in medium A and those incubated in medium B, and t is the time (in hours).

Initial rate of HCO₃⁻ influx was calculated by multiplying V by the buffering power measured for cells at that particular intracellular pH and was expressed in millimoles per liter of cells per hour. The DIDS-sensitive component of the HCO₃⁻ influx represents the activity of the Cl⁻-HCO₃⁻ anion exchanger. Because experiments were performed in the absence of Na⁺ in the incubation media, the DIDS-sensitive, Na⁺-independent HCO₃⁻ influx here measured represents the activity of the Na⁺-independent Cl⁻-HCO₃⁻ anion exchanger in erythrocytes.

Measurement of the Activity of the Na⁺-H⁺ Exchanger

The activity of the Na⁺-H⁺ exchanger was also assayed as the initial rate of change in intracellular pH after an acid load. To measure the activity of the Na⁺-H⁺ exchanger, we compared the rate of intracellular pH recovery in acid-loaded erythrocytes in the presence and ab-
The presence of Na⁺ (Na⁺ had been replaced with an equimolar amount of Ch⁺) as previously reported. The composition of the medium was (mmol/L) NaCl (or choline chloride), 150; MgCl₂, 1; CaCl₂, 1; glucose, 10; Tris-MOPS, 20; sucrose, 20; ouabain, 0.1; and bumetanide 0.025, pH 8, at 37°C. Both media were nominally HCO₃⁻ free, and 20 μmol/L DIDS was present in both of them. These were also the optimal conditions to promote the maximal activity of the exchanger.

Fig 2 illustrates the dependence of external Na⁺-dependent intracellular pH recovery on the incubation time. The initial recovery rate was calculated as described above for the Cl⁻-HCCV anion exchanger. The initial rate of Na⁺-dependent H⁺ efflux was calculated as the product of the initial rate of intracellular pH recovery on the addition of external Na⁺ and the buffering power and was expressed in millimoles per liter of cells per hour. This Na⁺-dependent H⁺ efflux was considered to represent the activity of the Na⁺-H⁺ exchanger in erythrocytes.

Chemicals
BCECF-AM was obtained from Molecular Probes, Eugene, Ore. Nigericin, acetazolamide, DIDS, ethacrynic acid, ouabain, bumetanide, Tris, MES, and MOPS were obtained from Sigma Chemical Co, St Louis, Mo; all other chemicals were from Merck Darmstadt, Germany.

Statistical Analysis
Values are expressed as means and range or mean±SEM. A Student's t test for single comparisons of quantitative parameters was used to assess statistical significance between normotensive control subjects and hypertensive patients. Confidence intervals were considered to assess statistical relevance of differences between subgroups of hypertensive patients. The correlation between continuously distributed variables was tested by univariate regression analysis.

Results
Characteristics of the Study Population
Table 1 shows the values obtained for the different clinical parameters in normotensive subjects and hypertensive patients. According to blood pressure levels, patients were considered to have mild to severe hypertension. According to hypertensive organ damage, patients were in stages I or II of the World Health Organization. No differences were observed in the different parameters measured between the two groups of subjects. The concentration of serum bicarbonate was similar in hypertensive patients (27.13±0.41 mmol/L) compared with normotensive subjects (26.21±0.41 mmol/L). Two normotensive subjects and four hypertensive patients exhibited values of serum bicarbonate above the upper normal limit of 30 mmol/L. Serum cholesterol levels were similar in hypertensive and normotensive individuals (5.72±1.15 vs 5.43±0.92 mmol/L, respectively). Thirteen patients, but none of the control subjects, exhibited echocardiographic criteria of left ventricular hypertrophy (left ventricular mass index >134 g/m² in men and >110 g/m² in women). Renal function tests were normal in all subjects.

Erythrocyte Measurements in Control Subjects
Fig 3 shows the intracellular pH values obtained in erythrocytes from 30 normotensive control subjects. It can be seen that intracellular pH ranged from 7.20 to 7.46 (mean, 7.33±0.01; Table 2). The reproducibility of the pH measurement was assessed by calculating the index of intra-assay variation in four samples (<1%) and the mean coefficient of interassay variation in six subjects (<1%). The buffering power ranged from 56 to 139 mmol/L per pH unit (mean, 89.02±4.00 mmol/L per pH unit; Table 2).

The activity of the Na⁺-independent Cl⁻-HCO₃⁻ anion exchanger measured in erythrocytes from normotensive subjects is shown in Fig 4. It can be observed that the DIDS-sensitive, Na⁺-independent HCO₃⁻ influx ranged from 7665 to 25 369 mmol/L cells per hour (mean, 15 629±897 mmol/L cells per hour; Table 2).

The measurement of the activity of the Na⁺-independent Cl⁻-HCO₃⁻ anion exchanger showed an index of intra-assay variation of 6% (n=4) and a mean coefficient of interassay variation of 13% (n=6).
The activity of the Na⁺-H⁺ exchanger measured in control subjects is presented in Fig 5. The Na⁺-dependent H⁺ efflux ranged from 14 to 553 mmol/L cells per hour (mean, 162±23 mmol/L cells per hour; Table 2). The measurement of this transporter had an index of intra-assay variation and a mean coefficient of interassay variation of 9% (n=4) and 15% (n=6), respectively.

Mean values of erythrocyte volume obtained in cells from control subjects are also included in Table 2. All values were within the range of reference values of our laboratory.

No significant differences in all the above measurements were observed between men and women or between subjects with and without a family history of hypertension. In addition, none of these parameters were influenced by age.

Erythrocyte Measurements in Hypertensive Patients

Table 2 gives the different parameters measured in erythrocytes from 35 patients with essential hypertension. Intracellular pH was diminished in hypertensive compared with control individuals (Table 2). Fig 3 shows individual values of intracellular pH in hypertensive patients. With the 100% confidence (lower) limit of the normotensive population as a cutoff point (7.19), it appears that a subgroup of 11 hypertensive patients had an abnormally low pH (mean, 7.15±0.01; range, 7.10 to 7.18). Intracellular pH was within the normal range in the remaining patients (mean, 7.33±0.02; range, 7.20 to 7.46). None of the patients with abnormal pH had been treated with captopril.

**Table 2. Erythrocyte Determinations in Normotensive Subjects and Hypertensive Patients**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Normotensive</th>
<th>Hypertensive</th>
</tr>
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<tbody>
<tr>
<td>Intracellular pH</td>
<td>7.33±0.01</td>
<td>7.27±0.02*</td>
</tr>
<tr>
<td>Buffering power (mmol/L per pH unit)</td>
<td>89.02±4.00</td>
<td>95.47±4.18</td>
</tr>
<tr>
<td>DIDS-sensitive, Na⁺-independent HCO₃⁻ influx (mmol/L cells per hour)</td>
<td>15 629±897</td>
<td>18 863±1081*</td>
</tr>
<tr>
<td>Na⁺-dependent H⁺ efflux (mmol/L cells per hour)</td>
<td>162±23</td>
<td>301±45†</td>
</tr>
<tr>
<td>Erythrocyte volume (μm³)</td>
<td>90.51±1.03</td>
<td>91.98±0.63</td>
</tr>
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</table>

DIDS, 4,4' diisothiocyanato stilbene-2,2'-disulfonic acid. Data are mean±SEM. *P<.05, †P<.005 vs normotensive subjects.
The activity of the Na\(^+\)-independent Cl\(^-\)-HCO\(_3^+\) anion exchanger was higher in erythrocytes from hypertensive patients than in erythrocytes from normotensive control subjects (Table 2). The individual values of the activity of anion exchange measured in hypertensive patients are shown in Fig 4. If the 100% confidence (upper) limit of the normotensive population is used as a cutoff point (25 316 mmol/L cells per hour), a subgroup of six hypertensive patients had an abnormally high anion exchanger (mean, 29 351 ± 1405 mmol/L cells per hour; range, 25 586 to 35 002 mmol/L cells per hour). The remaining patients exhibited values of the anion exchanger within the normal range (mean, 16 692 ± 812 mmol/L cells per hour; range, 9222 to 24 968 mmol/L cells per hour). Intracellular pH was lower (P < 0.05) in the six hypertensive patients with an abnormally increased anion exchanger (7.20 ± 0.03) than in hypertensive patients with a normal anion exchanger (7.28 ± 0.02). In addition, the activity of the anion exchanger was higher (P < 0.05) in hypertensive patients with abnormally low intracellular pH (20 806 ± 1200 mmol/L cells per hour) compared with hypertensive patients with normal intracellular pH (17 836 ± 956 mmol/L cells per hour).

The activity of the Na\(^+\)-H\(^+\) exchanger was increased in cells from hypertensive patients compared with cells from normotensive control subjects (Table 2). Values of the Na\(^+\)-H\(^+\) exchanger measured in hypertensive patients are depicted in Fig 5. With the 100% confidence (upper) limit of the normotensive population as a cutoff point (410 mmol/L cells per hour), it appears that a subgroup of 6 hypertensive patients had an abnormally high Na\(^+\)-H\(^+\) exchanger (mean, 668 ± 57 mmol/L cells per hour; range, 489 to 910 mmol/L cells per hour). The activity of the Na\(^+\)-H\(^+\) exchanger was within the normal range in the other 29 hypertensive patients (mean, 196 ± 27 mmol/L cells per hour; range, 44 to 378 mmol/L cells per hour). Intracellular pH was similar in patients with an abnormally increased Na\(^+\)-H\(^+\) exchanger (7.31 ± 0.02) than in patients with a normal Na\(^+\)-H\(^+\) exchanger (7.28 ± 0.02). On the other hand, no differences were found in the activity of the Na\(^+\)-H\(^+\) exchanger between hypertensive patients with abnormally diminished pH (296 ± 97 mmol/L cells per hour) and those with normal pH (302 ± 53 mmol/L cells per hour). Two hypertensive patients with abnormally increased activity of the Na\(^+\)-H\(^+\) exchanger also exhibited an abnormally high activity of the anion exchanger.

There were no significant differences in the buffering power or in the erythrocyte volume between the two groups of subjects (Table 2).

**Correlational Analysis**

The activity of the Na\(^+\)-independent Cl\(^-\)-HCO\(_3^+\) anion exchanger was inversely correlated with intracellular pH in all subjects (r = -0.33, P < 0.01). No statistically significant correlations were found between the activity of the Na\(^+\)-H\(^+\) exchanger and intracellular pH or erythrocyte volume.

A direct correlation was observed between the activity of the Na\(^+\)-independent Cl\(^-\)-HCO\(_3^+\) anion exchanger and serum HCO\(_3^+\) concentration in all subjects (r = 0.35, P < 0.01; Fig 6). A direct correlation was found between mean arterial pressure and the activity of the Na\(^+\)-independent Cl\(^-\)-HCO\(_3^+\) anion exchanger (r = 0.31, P < 0.05) in all subjects.

No other correlations were found between the activity of the Na\(^+\)-independent Cl\(^-\)-HCO\(_3^+\) anion exchanger and other clinical and biochemical parameters.

Intracellular pH was found to be inversely correlated with mean arterial pressure (r = -0.28, P < 0.05) in all subjects.

**Discussion**

In erythrocytes, the Na\(^+\)-independent Cl\(^-\)-HCO\(_3^+\) anion exchanger may exchange physiologically either extracellular Cl\(^-\) by intracellular HCO\(_3^+\) or intracellular Cl\(^-\) by extracellular HCO\(_3^+\). The exchange rate appears to be the same in the two modes of operation. Thus, because of several technical reasons (namely, fluorescence of BCECF was not linear at alkaline intracellular pH values, and we also measured the extrusion of H\(^+\) by the Na\(^+\)-H\(^+\) exchanger), we decided to measure the activity of the exchanger by determining DIDS-sensitive HCO\(_3^+\) influx in acidified erythrocytes. On the other hand, we have verified that this DIDS-sensitive HCO\(_3^+\) influx is not dependent on the presence of Na\(^+\) in the incubation medium. Therefore, we have considered that the DIDS-sensitive, Na\(^+\)-independent HCO\(_3^+\) influx here measured represents the activity of the erythrocyte Na\(^+\)-independent Cl\(^-\)-HCO\(_3^+\) anion exchanger.

The main finding of this study is that the activity of the erythrocyte Na\(^+\)-independent Cl\(^-\)-HCO\(_3^+\) anion exchanger was increased in hypertensive compared with control individuals. In addition, compared with values obtained in normotensive subjects, an abnormally high activity of the Na\(^+\)-independent Cl\(^-\)-HCO\(_3^+\) anion exchanger was observed in 6 (17%) hypertensive patients. Therefore, the above 6 patients were considered hypertensive, with an abnormally increased activity of the Na\(^+\)-independent Cl\(^-\)-HCO\(_3^+\) anion exchanger. The remaining 29 hypertensive patients did not exhibit this erythrocyte anion exchanger abnormality. Thus, we have considered these patients hypertensive with a normal Na\(^+\)-independent Cl\(^-\)-HCO\(_3^+\) anion exchanger. This observation confirms the heterogeneous nature of the alterations in mechanisms regulating cell pH present in essential hypertension.
We did observe that the activity of the Na\(^+\)-independent Cl\(^{-}\)-HCO\(_3\)\(^{-}\) anion exchanger in erythrocytes from normotensive subjects was not influenced by the family history of hypertension. This would suggest that the abnormality of the exchanger present in patients with hypertension is not linked to the genetic predisposition to the disease.

Several studies have described the effects of age and sex on the activity of erythrocyte transport systems. 25 However, we were unable to find any relation of these two parameters with the activity of the Na\(^+\)-independent Cl\(^{-}\)-HCO\(_3\)\(^{-}\) anion exchanger.

Gross changes in plasma lipids have been reported to be associated with changes in the ratio of cholesterol to phospholipid in the erythrocyte cell membrane and changes in anion transport. 26 More specifically, doubling the cholesterol content of the erythrocyte membrane inhibits the activity of the anion exchanger by approximately 50\%. 27 In this study, increased serum levels of cholesterol were found in only two patients with increased DIDS-sensitive, Na\(^+\)-independent HCO\(_3\)\(^{-}\) influx. The remaining four patients with this transport abnormality exhibited normal serum cholesterol. Thus, serum lipids appear not to be involved in the hyperactivity of the anion exchanger here observed.

It is known that in the mammalian erythrocyte there appears to be essentially no physiological regulation of band 3 protein that mediates the Cl\(^{-}\)-HCO\(_3\)\(^{-}\) anion exchanger. 28 Once the protein has been synthesized in the developing erythrocyte, the activity persists throughout the life of the cell. Thus, it could be suggested that an increased number of band 3 protein molecules may be involved in the increased activity of the anion exchanger. This possibility would be in agreement with the finding that the content of band 3 protein in erythrocyte membranes of spontaneously hypertensive rats is 15% to 20% greater than in normotensive control rats. 29 However, our results cannot distinguish among an increased number of transport units, an increased turnover of the transporter, or an abnormal interaction of the transporter with intracellular HCO\(_3\)\(^{-}\).

A second finding of this study is that the basal erythrocyte pH was diminished in hypertensive patients compared with control subjects. This confirms previous results of decreased intracellular pH (measured by nuclear magnetic resonance spectroscopy) in erythrocytes from humans and rats with untreated hypertension. 12 A diminished pH also has been observed in lymphocytes from spontaneously hypertensive rats 30,31 and in cultured vascular smooth muscle cells from stroke-prone spontaneously hypertensive rats. 32 However, other discrepant results on intracellular pH have been reported in both human \(^1\text{-}11\) and experimental \(^13\text{-}28\) hypertension. These conflicting observations suggest that pH regulation in hypertension may be differently affected depending on the cell type, the animal model, the phase of the hypertensive process, and methodological aspects. Nevertheless, because intracellular pH has an important function in the regulation of intracellular events, it is reasonable to assume that any deviation from normality may have adverse consequences for the cell.

What is the origin of a diminished pH in erythrocytes of hypertensive patients? Three observations suggest that an association might exist between increased activity of the Na\(^+\)-independent Cl\(^{-}\)-HCO\(_3\)\(^{-}\) anion exchanger and diminished intracellular pH in erythrocytes of hypertensive patients: (1) Intracellular pH was inversely correlated with the activity of the Na\(^+\)-independent Cl\(^{-}\)-HCO\(_3\)\(^{-}\) anion exchanger in the whole group of subjects studied; (2) intracellular pH was more diminished in those patients with abnormally increased anion exchanger than in the remaining patients with normal anion exchanger; and (3) the activity of the anion exchanger was higher in the subgroup of hypertensive patients with abnormally low intracellular pH than in the subgroup of hypertensive patients with normal intracellular pH. However, we are aware that we assessed the activity of the anion exchanger at intracellular pH values different from the basal intracellular pH. Thus, we do not assume that the increased activity of the anion exchanger is necessarily responsible for the diminished pH.

Nevertheless, the study of the activation of the Na\(^+\)-independent Cl\(^{-}\)-HCO\(_3\)\(^{-}\) anion exchanger by an outward H\(^+\) gradient provides an assessment of functional properties such as the capacity and ability of the exchanger to regulate cytosolic pH in response to deviations of basal pH. Therefore, the above association led us to propose that the regulatory role of the Na\(^+\)-independent Cl\(^{-}\)-HCO\(_3\)\(^{-}\) anion exchanger in maintaining a normal erythrocyte pH may be altered in those hypertensive patients with diminished intracellular pH. Alternatively, other mechanisms also could be involved in a hypothetical disregulation of the intracellular pH in those hypertensive patients with diminished erythrocyte pH. These mechanisms include (1) increased intracellular H\(^+\) production secondary to enhanced entry of CO\(_2\) emerging from the tissues, a possibility that would be in keeping with an increased activity of the erythrocyte Cl\(^{-}\)-HCO\(_3\)\(^{-}\) anion exchanger in the conversion of CO\(_2\) to HCO\(_3\)\(^{-}\) in blood 37; (2) intracellular accumulation of H\(^+\) as a result of an increased activity of the erythrocyte Ca\(^{2+}\)-H\(^+\) exchange, a mechanism primarily involved in the control of intracellular Ca\(^{2+}\) content 36; and (3) a physicochemical effect such as Donnan equilibrium. 18 The first possibility is in agreement with our finding that a direct correlation exists between the activity of the anion exchanger and serum concentration of HCO\(_3\)\(^{-}\).

Another finding of this study is that the activity of the erythrocyte Na\(^+\)-H\(^+\) exchanger was increased in hypertensive patients compared with control subjects. This is in agreement with results previously reported in erythrocytes 15,40,41 and other circulating cells 41,42,43 of essential hypertensive patients.

The Na\(^+\)-H\(^+\) exchanger is an important mechanism for the regulation of intracellular pH and cell volume. 44 However, we were unable to find any association between the activity of the Na\(^+\)-H\(^+\) exchanger and either intracellular pH or erythrocyte volume. Although we recognize that the activity of the exchanger was measured in conditions that do not correspond to those basal conditions reflected by cell pH and volume, it is tempting to speculate that overactivity of the Na\(^+\)-H\(^+\) exchanger present in erythrocytes of hypertensive patients is not linked to alterations of these two parameters.

Among the possible explanations for the increased activity of the erythrocyte Na\(^+\)-H\(^+\) in hypertension, Canessa et al 44 have proposed a higher number of
exchanger sites or abnormal exchanger regulation by phosphorylation. Alternatively, it has been shown that the erythrocyte Na+-H+ exchanger is stimulated by elevation of intracellular Ca2+.46 Because it has been demonstrated that cytosolic calcium concentration is increased in erythrocytes from hypertensive patients,46 the possible role of this alteration in the hyperactivity of the erythrocyte Na+-H+ exchanger in hypertension deserves to be considered.

Despite the correlations found here between arterial pressure and the activity of the anion exchanger and intracellular pH, we are aware that extrapolation of data from cells other than vascular smooth muscle to the pathophysiology of hypertension is necessarily speculative. It is in this regard that we mention that in experiments conducted with human subcutaneous resistance arteries, Izzard et al7 have found that basal intracellular pH is not different in vessels from hypertensive patients compared with those from normotensive control subjects and that there are no differences in the activities of the Na+-H+ exchanger and of the anion exchanger between the two groups of subjects. However, as Izzard et al7 note, because an intact resistance vessel is a composite of cell types, a difference in vascular smooth muscle cell pH could be missed in vessels from patients with essential hypertension. On the other hand, as the same authors recognize,7 to examine the Na+-H+ exchanger and anion exchanger in physiological conditions (ie, at basal intracellular pH), the transporters are blocked and the effect on intracellular pH recorded. Thus, this methodology does not rule out the possibility of subtle differences in exchanger activities in vessels from hypertensive patients compared with those from control subjects. Other methods of assessing these exchangers—ie, measurement of the initial rate of recovery from an acid load, as we did in this study—may be more sensitive for the detection of small variations of activity between control subjects and patients.

Thus, we believe that our finding of increased activity of the Na+-independent Cl–/HCO3– anion exchanger in association with reduced intracellular pH in erythrocytes from essential hypertensive patients may provide additional insights on some alterations of vascular functions (ie, vasoconstriction, abnormal growth) in hypertension. This is further supported by previous suggestions that this transport mechanism could be an important factor in maintaining vascular tone20 and in transducing growth factor signals.48

In conclusion, we have confirmed that cell pH is diminished and the Na+-H+ exchanger is increased in erythrocytes of essential hypertensive patients. In addition, our data have shown a new abnormality of erythrocyte pH regulation in hypertension consisting of an excessive activity of the Na+-independent Cl–/HCO3– anion exchanger. Moreover, we found that hyperactivity of the anion exchanger is associated with diminished erythrocyte pH in a subgroup of essential hypertensive patients. Therefore, it could be postulated that participation of the anion exchanger in erythrocyte pH regulation is compromised in those hypertensive patients with diminished pH. This observation could be of interest to further understand the cellular basis of the hypertensive process.
Erythrocyte anion exchanger activity and intracellular pH in essential hypertension.
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