Effects of Diltiazem on Cation Transport Across Erythrocyte Membranes of Hypertensive Humans

FARHAD KHALIL-MANESH, KALYANASUNDARAM VENKATARAMAN, DINESH R. SAMANT, AND UDAY G. GADGIL

SUMMARY The effects of the calcium antagonist diltiazem on diastolic blood pressure and various parameters of erythrocyte membrane cation transport were evaluated in hypertensive patients with diastolic blood pressure between 95 and 110 mm Hg in a placebo-controlled, double-blind parallel study. Twenty-one patients completed the study; 13 received placebo, while 8 received diltiazem. Diastolic blood pressure, intracellular sodium and calcium concentrations, ouabain-sensitive Na⁺,K⁺-adenosine triphosphatase (ATPase) activity, and net sodium efflux and potassium influx across red blood cell membranes were examined in both groups at the end of placebo run-in, at the end of the titration phase, and at the completion of study. In the placebo group, none of the parameters changed significantly. In the drug-treated group, diastolic blood pressure declined by approximately 10% (placebo, 95.1 ± 8.9; drug-treated, 86.9 ± 4.9 mm Hg; p<0.03) at the end of the study. There were also reductions in intracellular sodium (placebo, 7.9 ± 1.8; drug-treated, 5.2 ± 0.4 mmol/L cells; p<0.002) and calcium (placebo, 13.5 ± 1.6; drug-treated 10.8 ± 3.3 μmol/L cells; p<0.03) concentrations, accompanied by a simultaneous rise in the activity of the ouabain-sensitive Na⁺,K⁺-ATPase of erythrocyte membranes (placebo, 7.1 ± 1.1 x 10⁻¹; drug-treated, 9.0 ± 0.6 x 10⁻² μM inorganic phosphate/hr/mg; p<0.001) at the end of the study. However, no significant change in the ouabain-insensitive moiety of the ATPase pump was found. Diltiazem treatment increased net sodium efflux and potassium influx. It is concluded that diltiazem reduces diastolic blood pressure of hypertensive subjects. Erythrocyte studies indicate that diltiazem not only blocks entrance of calcium into the cells but may also stimulate Na⁺,K⁺-ATPase activity, resulting in reduction in intracellular sodium concentration, thus suggesting a possible dual mechanism for its antihypertensive effects.

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KEY WORDS • ouabain-sensitive ATPase • cation fluxes • erythrocyte • diltiazem • hypertension • calcium channel blockers

ERYTHROCYTES from normal and hypertensive humans have been a valuable source for the study of cellular transport processes in hypertension¹⁻³ because they are easily obtainable and sufficiently resistant to in vitro manipulation. Several abnormalities of sodium transport across the erythrocyte membrane have been shown in persons with essential hypertension, including active Na⁺ and K⁺ fluxes,¹,² furosemide-sensitive Na⁺⁺K⁺ cotransport,³ and Na⁺⁻Li⁺ countertransport.⁴ An increase in white blood cell sodium concentration related to a reduction in the ouabain-sensitive component of the Na⁺⁻K⁺ pump has also been described.⁵⁻⁸

In the present investigation, we studied the changes that occur in the erythrocytes of hypertensive persons treated with diltiazem to test the hypothesis that the abnormalities in the red blood cell cation concentrations and various pump activities are reversed in those who respond to the drug treatment. These studies were conducted in a placebo-controlled, double-blind, parallel fashion.

Patients and Methods
Selection of Study Patients
The protocol was approved by the institutional review board, and written informed consent was obtained from each subject. Twenty-one patients, 12 men (average age, 51 ± 15 years) and 9 women (average age, 57 ± 13 years), completed the protocol. Inclusion criteria were 1) a stable resting supine diastolic blood pressure (DBP) between 95 and 110 mm Hg at least 2 weeks after cessation of antihypertensive medications and 2) a stable DBP with variation of 7 mm Hg or less.
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between two consecutive weekly measurements starting with days 7 through 10 of the placebo run-in.

After a 2-week placebo run-in phase, the eligible patients were randomized to placebo or slow-release diltiazem treatment in a double-blind parallel fashion. Patients were titrated to the optimum dose of the blinded medication for up to 6 weeks. Doses for the first two weeks were 120 mg of diltiazem or placebo, 240 mg for the second 2 weeks, and 360 mg for the third 2 weeks. Titration was continued until a 10% decline of supine diastolic pressure was achieved or until the end of the titration period. Once a final dose had been determined, patients were entered into a 4-week drug evaluation period. Blood was drawn on three occasions for cellular studies: 1) at the end of placebo run-in, 2) at the end of titration period, and 3) at the end of the study period.

Measurement of Intracellular Electrolytes

Intracellular sodium concentrations were measured according to the method of Guthe et al. Heparinized blood (3 ml) was centrifuged at 1750 g for 5 minutes at 4°C, the plasma anduffy coat were removed, and the erythrocytes were washed five times with approximately 20 ml of ice-cold 110 mM MgCl₂. Hematocrit was adjusted to approximately 50% following the last wash, and the hematocrit of the suspension was measured in triplicate. The red blood cells were then lysed in lithium diluent, and their intracellular Na⁺ was measured on a flame photometer.

Total cell calcium content was measured by atomic absorption spectroscopy of a dry-ashed sample of red blood cells. Red blood cells (3 ml) were washed three times in 150 mM NaCl plus 1 mM EGTA at 4°C. Cells were then hemolyzed in 0.01 N NH₄OH, and a sample of the hemolysate was added to a silica crucible, evaporated to dryness under an infrared lamp, and combusted at 500°C in a muffle furnace. The residue was extracted with 3 ml of 0.1 N HCl plus 10 mM SrCl₂ with shaking for 2 hours at room temperature. The contents of the crucible were decanted into polypropylene tubes, and the ion oxide was removed. The calcium concentration in the supernatant was determined on an atomic absorption spectrophotometer (Perkin-Elmer, Norwalk, CT, USA) at 422.6 nm.

All measurements were performed in duplicate. Special care was taken during calcium determinations to prevent contamination from the environment. The atomic absorption spectrophotometer was calibrated before each set of measurements, and a control sample was used to allow for daily instrument fluctuations. Duplicate readings for calcium in the samples did not vary more than 6%.

Preparation of Erythrocytes for Cation Flux Measurements

The active Na⁺ extrusion and K⁺ uptake by intact erythrocytes were determined using p-chloromercuribenzenesulfonate (PCMB) to load the cells. Fresh heparinized blood samples (5 ml) were centrifuged at 1750 g for 5 minutes at 4°C, the plasma anduffy coats were removed, and the erythrocytes were washed three times with 150 mM ice-cold NaCl. Cells were incubated in the loading medium (150 mM NaCl, 1 mM MgCl₂, 2.5 mM Na₂HPO₄, and 0.1 mM PCMB, pH 7.4) to a final hematocrit of 8% for 20 hours at 4°C, with a medium change after 4 to 5 hours.

For sodium and potassium flux determinations, packed erythrocytes were incubated in a flux medium (145 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 2.4 mM Na₂HPO₄, 10 mM glucose, and 4 mM cysteine, pH 7.4) at a 5% hematocrit for 1 hour at 37°C to remove the PCMBs. After centrifugation at 1750 g at 4°C for 15 minutes, the cells were washed and finally resuspended in the same flux medium without cysteine to a 10% hematocrit. Aliquots (500 µl) of this suspension were poured into tubes containing 500 µl of flux medium with and without 0.2 mM ouabain. Samples were incubated at 37°C for 0, 1, 2, and 3 hours. At the end of each incubation period, cells were separated and washed with three changes of 150 mM choline chloride by centrifugation at 1750 g at 4°C for 5 minutes.

Measurement of Cation Fluxes

Sodium and potassium contents of erythrocytes were prepared and measured as described earlier using a flame photometer. Hemoglobin content was determined spectrophotometrically at 541 nm. The Na⁺ and K⁺ contents of the packed cells were expressed as millimoles per liter of cell water, calculated on the basis of hemoglobin content and a water content of 70% (vol/vol). Treatment with PCMB resulted in an increase of approximately 8% in the water content of the cells. The cation contents were corrected for this increased water content.

There were no nonspecific effects of diltiazem on the transport processes. In an in vitro study, diltiazem did not interfere with electrolyte determinations or with the activity of a partially purified Na⁺-K⁺-adenosine triphosphatase (ATPase; Sigma, St. Louis, MO, USA).

Preparation of Erythrocyte Membranes for ATPase Assays

Erythrocytes (5 ml) were washed with 25 ml of 0.25 M NaCl solution and centrifuged at 1750 g for 15 minutes at 4°C. Washed cells were lysed by freezing and thawing them several times. The resulting hemoly-sates were then washed five times with a solution containing 0.02 M NaCl and 5 mM Tris at pH 7.4. The resulting pellets were washed once more with distilled water, resuspended in a small volume of water, lyophilized, and stored at −20°C until required.

Measurement of Na⁺,K⁺-ATPase of Erythrocytes

The ATPase activity was assayed in a medium containing 0.3 to 0.5 mg of erythrocyte per milliliter, 50 mM Tris (pH 7.4 at 37°C), 6 mM MgCl₂, 100 mM NaCl, 20 mM KCl, 6 mM adenosine 5'-triphosphate (ATP) and 0.5 mM EGTA and a medium containing the same components plus 0.2 mM ouabain. The difference between the two assays was the activity of ouabain-sensitive Na⁺,K⁺-ATPase. The ATPase incubations were done at 37°C for 90 minutes, and reac-
tions were stopped by the addition of 0.5 ml of ice-cold 8.6% trichloroacetic acid. Inorganic phosphate (P) released during the hydrolysis of ATP to adenosine 5′-diphosphate (ADP) was measured colorimetrically according to the method of Fiske and Subbarow.14

Statistical Analysis

Statistical evaluation of the results were performed by paired *t* tests between two different phases of the study in the same group, unpaired *t* tests between placebo and drug groups in the same study phase, and repeated-measures analysis of variance using the BMDP statistical software package.15 Data are presented as means ± SD.

Results

Of the 21 randomized patients who completed the study, 13 received placebo and 8 received diltiazem. The majority of subjects in both placebo and drug-treated groups were white, and there was no significant demonstrable difference between the two groups.

The results obtained for DBP are shown in Figure 1. There was an approximately 10% decline in DBP of diltiazem-treated patients when compared with placebo-treated patients during the study. However, maximum reduction in DBP was observed at the end of titration period.

Changes in red blood cell sodium ([Na]+) and calcium ([Ca]+) are shown in Figures 2 and 3, respectively. The mean cell sodium contents at the end of the study for placebo and drug-treated groups were 7.9 ± 1.8 and 5.2 ± 0.4 mmol/L cells, respectively (*p*<0.002). There was an overall reduction in [Na]+ in the drug-treated group during the study, while the same parameter for the placebo group did not change significantly. Intracellular calcium was also lower in the drug-treated group (10.8 ± 3.3 μmol/L cells) than in the placebo group (13.5 ± 1.6 μmol/L cells; *p*<0.03) at the end of the drug evaluation (see Figure 3).

Results obtained for ouabain-sensitive Na+,K+-ATPase activity in fragmented erythrocyte membranes are given in Table 1. This parameter increased in the drug-treated group from 6.4 ± 0.5 × 10−2 μM P/hr/mg protein during placebo run-in to 9.0 ± 0.6 × 10−2 μM P/hr/mg protein at the end of the drug evaluation phase. A similar increase was not observed in the placebo group. There was, however, no significant change in the ouabain-insensitive moiety of the ATPase activity in the drug-treated group, as compared with the placebo group (see Table 1).

The active cation fluxes were determined after treatment of the erythrocytes with PCMB, during which cells lost K+ and accumulated Na+ (Table 2). Both sodium efflux and potassium influx of intact erythrocytes increased significantly after diltiazem treatment.
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Table 1. Erythrocyte Membrane ATPase Activity

<table>
<thead>
<tr>
<th>Study phase</th>
<th>Placebo (n = 13)</th>
<th>Diltiazem (n = 8)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Ouabain-sensitive</td>
<td>Ouabain-insensitive</td>
</tr>
<tr>
<td>Placebo run-in</td>
<td>6.4 ± 0.5</td>
<td>5.2 ± 0.3</td>
</tr>
<tr>
<td>Titration</td>
<td>6.9 ± 1.1</td>
<td>5.0 ± 0.5</td>
</tr>
<tr>
<td>Drug evaluation</td>
<td>7.1 ± 1.1</td>
<td>5.0 ± 0.3</td>
</tr>
</tbody>
</table>

Values are means ± SD. *p < 0.001, compared with placebo run-in period.

Table 2. Net Sodium and Potassium Fluxes and Cation Content After p-Chloromercuribenzenesulfonate Treatment

<table>
<thead>
<tr>
<th>Study phase</th>
<th>Placebo (n = 13)</th>
<th>Diltiazem (n = 8)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Na⁺ efflux</td>
<td>K⁺ influx</td>
</tr>
<tr>
<td></td>
<td>(mmol/L RBCs/hr)</td>
<td>(mmol/L RBCs/hr)</td>
</tr>
<tr>
<td>Placebo run-in</td>
<td>3.1 ± 0.2</td>
<td>1.9 ± 0.2</td>
</tr>
<tr>
<td>Titration</td>
<td>3.2 ± 0.5</td>
<td>2.1 ± 0.4</td>
</tr>
<tr>
<td>Drug evaluation</td>
<td>3.3 ± 0.3</td>
<td>2.2 ± 0.4</td>
</tr>
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</table>

Values are means ± SD. RBC = red blood cell. *p < 0.005, †p < 0.01, compared with placebo run-in period.

The cation content of cells after PCMBS treatment is also shown in Table 2. There was no statistically significant increase in the cation content of the cells after PCMBS treatment, indicating that the postload sodium concentration did not vary, affecting cation fluxes.

As shown in Figure 4A, there was a significant positive correlation between intracellular sodium concentration and DBP (r = 0.81, p < 0.001). There was also a strong but inverse correlation between the ouabain-sensitive part of the ATPase and DBP (r = −0.78, p < 0.001) in the drug-treated group (Figure 4B).

One of the subjects in the drug group did not improve during the study. His DBP as well as all other cellular parameters either increased or remained unchanged throughout the study period. The data for this patient were not included in the statistical analysis as he was found to be an outlier by using the BMDP statistical software package.

Discussion

The present study indicates that the diltiazem-induced reduction of DBP in hypertensive persons is accompanied by an overall reduction in intracellular sodium and calcium and an increase in ouabain-sensitive Na⁺,K⁺-ATPase activity, as well as in cation fluxes.

Increased [Na⁺] has been reported previously in erythrocytes from hypertensive patients. The [Na⁺] of our hypertensive subjects was 8.8 ± 1.7

Figure 4. Positive correlation (A) between intracellular sodium concentration ([Na⁺]) and DBP, and inverse correlation (B) between ouabain-sensitive Na⁺,K⁺-ATPase and DBP in hypertensive subjects treated with diltiazem during placebo run-in phase (C), titration phase (D), and drug evaluation phase (E). Pᵢ = inorganic phosphate.
mmol/L red blood cells and compares favorably with values reported by other investigators (8.3 ± 1.0 mmol/L red blood cells).1 On the other hand, Canessa et al.failed to show significant differences in sodium content of red blood cells between hypertensive and normotensive subjects. However, careful scrutiny of their data reveals that the normotensive subjects had significantly higher [Na] than the values reported by other groups.

In the present study, red blood cell sodium concentration of hypertensive subjects treated with diltiazem was lowered significantly, showing a positive correlation with DBP (see Figure 4A). Similar lowering of erythrocyte [Na], was shown for hypertensive patients whose blood pressures were controlled with thiazide diuretics in combination with a vasodilator or a sympathetic blocking agent, or both.1 Also, increased sodium content in lymphocytes of hypertensive subjects has recently been shown to be reduced with thiazide diuretics and captoril. Ambrosioni et al. showed a somewhat weaker positive correlation (r = 0.55) between [Na], of lymphocytes with blood pressures of hypertensive subjects.

Increased intraerythrocyte sodium concentration has been suggested to result from reduced active sodium extrusion in sustained essential hypertension.17,20 Our data (see Table 2) indicate that patients in the diltiazem-treated group had a lower initial net sodium efflux rate that was increased after diltiazem administration. This increase was accompanied by increased potassium influx. The changes observed in cation fluxes in the drug-treated group were accompanied by an increase in the activity of the ouabain-sensitive part of the ATPase; the ouabain-insensitive moiety remained unchanged throughout the study. In contrast, Garay and colleagues only implicated outward Na⁺-K⁺ co-transport for the reduction in sodium efflux. Others, however, have been unable to confirm their findings.

The average baseline value for ouabain-sensitive Na⁺,K⁺-ATPase of hypertensive subjects (6.8 ± 1.0 × 10⁻² μM P/hr/mg protein) agrees with that of other investigators. In contrast, Garay et al. and Dagher and Garay have suggested an increase in the Na⁺,K⁺-ATPase activity of hypertensive subjects that was thought to compensate for a reduction in the activity of the co-transport system. However, this suggestion has been disputed by Cole1 and Swarts et al., who observed no increase in ouabain-sensitive Na⁺,K⁺-ATPase activity in hypertensive subjects.

One of the important observations in our study relates to a significant increase in the ouabain-sensitive moiety of the ATPase complex accompanied by a decrease in [Na], after treatment with diltiazem. Some of the previous investigators, despite finding a reduction in [Na],, failed to demonstrate a change in the ouabain-sensitive ATPase activity.1,8,11,19 On the other hand, it has recently been shown that the total sodium efflux of salt-sensitive Dahl rat fed excess sodium is markedly reduced but becomes normal with nifedipine treatment. This phenomenon suggests that Na⁺-K⁺ pump inhibition may be involved in the development of Dahl rat hypertension and that it is prevented by chronic nifedipine treatment. Thus, the stimulation of the ATPase activity with diltiazem may be shared with other calcium channel blockers, and the possible mechanisms are discussed below.

A reduction in intracellular calcium concentration was not surprising since diltiazem is a calcium channel blocker. Similar effects on calcium transport in the vascular muscle resulting in vasodilatation would explain its antihypertensive effects. However, the stimulation in ouabain-sensitive Na⁺,K⁺-ATPase accompanied by a reduction in [Na], may decrease basal vascular reactivity, as well as the sensitivity to other circulating factors, such as catecholamines.

The correlations between [Na], and the ouabain-sensitive ATPase with DBP were strong in the diltiazem-treated group. But, it is not clear whether the cellular changes that occurred with diltiazem treatment were primary effects of the drug or were secondary to the lowering of the blood pressure. We, however, speculate that 1) the inhibition of calcium influx with diltiazem may alter the intracellular ratio of sodium to calcium, which in turn may activate the ouabain-sensitive Na⁺,K⁺-ATPase as a compensatory mechanism, and 2) changes in the membrane conformation caused by diltiazem binding could directly stimulate the ouabain-sensitive pump activity. Recently, Sasaki et al. showed that diltiazem improved the structural deformability of rat erythrocytes. Rachkov and Pashukov have also shown that reduced ATPase activity of rabbit erythrocytes with vasorelaxant hypertension is normalized with vasodilatory drugs such as papaverine, pentamin, and nicotinic acid, and this may also be true of diltiazem. Other investigators have suggested that diltiazem, nifedipine, verapamil, and nimodipine also have stimulatory effects on the ouabain-sensitive Na⁺,K⁺-ATPase. However, there is no uniform agreement in the literature about the effects of calcium channel blockers on Na⁺,K⁺-ATPase activity, probably because of differences in experimental conditions and the dosage of the drugs used.

In summary, our study shows that the diltiazem-induced reduction in DBP of hypertensive subjects is accompanied by a lowering of intraerythrocyte calcium concentration and a possible stimulation of the activity of ouabain-sensitive Na⁺,K⁺-ATPase and a reduction in [Na]. We conclude that diltiazem may similarly block entry of calcium into the vascular smooth muscle cells while stimulating their ouabain-sensitive pump activities, resulting in lower cell sodium. Thus, we suggest a possible dual mechanism for the antihypertensive effect of diltiazem.

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References

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