Enhanced Calcium-Sensitivity of Erythrocytes in Hypertension

—Calcium-induced Changes of Erythrocyte Osmotic Fragility in Essential Hypertension—

KAZUSHI TSUDA, M.D., YOHSUKE MINATOGAWA, M.D., SEIKO FURUYA, M.D.
EIZO UEDA, M.D., YOSHIKO KUSUYAMA, M.D., HIROKI SHIMA, M.D.
TOSHIKI TAMAKI, M.D., ICHIRO NISHIO, M.D., RYO KIDO, M.D.
AND YOSHIKI MASUYAMA, M.D.

To investigate the Ca-sensitivity of the erythrocyte membrane in hypertension, the changes of the osmotic fragility of erythrocytes by Ca-loading and the effects of Ca-channel blockers or calmodulin-antagonist were observed in patients with essential hypertension.

Erythrocytes were obtained from untreated patients with essential hypertension and age-matched normotensive subjects. Treatment of erythrocytes with Ca-ionophore A23187 and Ca in bathing medium caused the reduction of the osmotic fragility of erythrocytes dose-dependently on Ca-concentration. The degree in alteration of the osmotic fragility of erythrocytes was greater in essential hypertension than that in normotensive controls. In addition, Ca-induced changes of erythrocyte osmotic fragility was inversely correlated with the plasma renin activity in essential hypertension.

In the presence of Ca-antagonists (verapamil, diltiazem) or calmodulin-antagonist (trifluoperazine), the reduction of the osmotic fragility of erythrocytes by Ca-loading was inhibited, and the differences of the osmotic fragility of erythrocytes between the hypertensives and the normotensive controls were abolished by these drugs.

These results suggest that the greater changes of the osmotic fragility of the erythrocytes by Ca-loading in essential hypertension might be due to the abnormality of Ca-handling of the cell membranes causing an increase in the intracellular Ca concentration, contributing at least partially to the pathogenesis of hypertension.

RECENTLY, it has been proposed that the biochemical and biophysical abnormalities of the cell membranes might be important factors in the etiology of hypertension. Widespread membrane abnormalities of the cells present in many organ system, including circulating blood cells. The alterations of intracellular Ca or Na concentration in the blood cells from hypertensive subjects have been already reported1−3 and they have been supposed to be due to the changes of the membrane ionic transport4−5.

On the other hand, an increase in Ca-contents of erythrocytes can induce characteristic changes,

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Ca-antagonists
Calmodulin-antagonist
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Hypertension

Division of Cardiology, Department of Medicine, *Department of Biochemistry, Wakayama Medical College, Wakayama, Japan

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Mailing address: Kazushi Tsuda, M.D., Division of Cardiology, Department of Medicine, Wakayama Medical College, Wakayama 640, Japan

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such as reduced osmotic fragility, decreased deformability, echinocyte formation or ATP-depletion in cells. Some of these phenomena may be induced by the binding of the intracellular Ca to cytoskeleton proteins distributed in the erythrocyte membranes. It is anticipated that these cytoskeleton proteins resemble the contractile proteins in the vascular smooth muscle cells or in other tissues. So, it is likely that the relationship between Ca and erythrocyte membranes represents Ca-handling of the cell membranes.

In this study, we examined Ca-induced changes of the osmotic fragility of erythrocytes taken from the patients with essential hypertension, and investigated the Ca-sensitivity of the cell membranes in hypertension. In addition, the effects of Ca-antagonists or a calmodulin-antagonist on the Ca-induced changes of erythrocyte osmotic fragility were studied in hypertension.

MATERIALS AND METHODS

Forty-three patients with essential hypertension, 25 males and 18 females (age: 44.2 ± 1.8 years old, mean ± SEM, blood pressure: 172.4 ± 3.3/104.1 ± 1.7 mmHg) and age-matched 33 normotensive subjects, 23 males and 10 females (age: 44.9 ± 3.2 years old, blood pressure: 113.6 ± 1.5/70.0 ± 2.1 mmHg) were studied. The hypertensive patients were in stage I or II of WHO classification of hypertension and had no medication for at least two weeks before the study. The levels of serum protein, cholesterol, triglycerides, sodium or potassium were not significantly different between the hypertensive patients and the normotensive controls.

1. Determination of the osmotic fragility of erythrocytes

Blood was obtained by venous puncture into the heparinized tube after 30-minute bed rest. Erythrocytes were washed three times with physiological saline, and incubated for 30 minutes in the buffer (140 mM-NaCl, 20 mM-Tris-HCl, pH 7.4, 37°C), containing Ca-ionophore A23187 (0.9 µM) and CaCl₂ (0–20 mM). One hundred microliters of packed erythrocytes were dissolved in 200 µl of the buffer.

The osmotic fragility of erythrocytes was estimated by the coil planet centrifuge method. Ten microliters of the incubated solution were applied to the high osmotic end of the plastic coil (0.3 mm in diameter and 3 m in length), in which the osmotic gradient had been prepared from 150 milliosmol (mOsm) to 30 mOsm by NaCl. The coil was incubated at 37°C for 10 minutes, and centrifuged for 10 minutes at 1600 rotations per minute with 16 self-rotations per minute by the coil planet centrifuge system (CPC, Type-SP, Iatron, Tokyo, Japan).

Erythrocytes were driven up through the coil in gradients of NaCl from 150 to 30 mOsm, and hemolyzed where their membranes could not resist the disruptive force of hypopsmotic stress. The distribution of the hemoglobin from the ruptured erythrocytes was determined by the densitometer (DMU 33-C, Toyo Kagaku Sangyo Co., Ltd., Tokyo Japan) at 565 nm. The osmotic fragility of erythrocytes was expressed as milliosmol of NaCl at hemolysis starting point (HSP), hemolysis maximum point (HMP) and hemolysis end point (HEP). HSP and HMP were sometimes difficult to determine exactly. So we adopted HEP as an indicator of the osmotic fragility of erythrocytes.

2. Effects of Ca-antagonists (verapamil or diltiazem) and calmodulin antagonist on Ca-induced changes of the osmotic fragility of erythrocytes.

To investigate the effects of Ca-antagonists and calmodulin antagonist on Ca-induced changes of the osmotic fragility of erythrocytes, 100 µl of packed erythrocytes were preincubated with 50 µl of the buffer containing Ca-antagonists (140 mM-NaCl, 20 mM-Tris-HCl, pH 7.4, containing verapamil 5.0 × 10⁻⁴ – 1.0 × 10⁻³ M, or diltiazem 5.0 × 10⁻⁴ – 1.0 × 10⁻³ M) or calmodulin antagonist (trifluoperazine 1.0 × 10⁻³ M).

After three hour incubation at 37°C, Ca-ionophore A23187 (0.9 µM) and CaCl₂ (1.0 mM), in the 200 µl of the buffer, were added, and Ca-loading to erythrocytes was performed in the presence of Ca-antagonists (final concentration of verapamil 7.1 × 10⁻⁵ M and 1.4 × 10⁻⁴ M; diltiazem 7.1 × 10⁻⁵ M and 1.4 × 10⁻⁴ M) or trifluoperazine (1.4 × 10⁻⁴ M). Then, the solution was incubated for 30 minutes at 37°C, and the osmotic fragility was determined by coil planet centrifuge system.

3. Measurement of Plasma renin activity (PRA)

PRA of the same samples were estimated by radioimmunoassay (AI formation: ng/ml/hr).

Values are expressed as mean ± SEM. Statistical significance was determined by Student’s t-
Fig.1 Alterations of the osmotic fragility of erythrocytes by Ca-loading determined by coil planet centrifuge system. Ca-loading to erythrocytes was performed in the buffer (NaCl 140 mM, Tris-HCl 20 mM, pH 7.4, 37°C) containing Ca-ionophore A23187 and CaCl₂. HSP = hemolysis starting point; HMP = hemolysis maximum point, and HEP = hemolysis end point.
(1) A23187 0.9 µM, CaCl₂ (-)
(2) A23187 0.9 µM, CaCl₂ 0.5 mM
(3) A23187 0.9 µM, CaCl₂ 1.0 mM
(4) A23187 0.9 µM, CaCl₂ 2.0 mM

<table>
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<td>CaCl₂</td>
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<td>1.0 mM</td>
<td>2.0 mM</td>
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<tr>
<td>HT</td>
<td>78.8 ± 0.9***</td>
<td>60.7 ± 3.2</td>
<td>46.9 ± 2.4**</td>
<td>48.1 ± 3.2</td>
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<tr>
<td>NT</td>
<td>71.4 ± 1.1</td>
<td>64.1 ± 2.1</td>
<td>56.1 ± 2.0</td>
<td>50.9 ± 2.2</td>
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<td>(n = 25)</td>
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Values are expressed as milliosmol of NaCl at hemolysis end point (HEP) determined by coil planet centrifuge system. (mean ± SEM, **p < 0.005, ***p < 0.001)

**TABLE Ib**  
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<th>0.9 µM</th>
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<td>CaCl₂</td>
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<tr>
<td>HT</td>
<td>76.6 ± 3.6%**</td>
<td>46.1 ± 2.4%***</td>
<td>58.0 ± 3.1%*</td>
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<tr>
<td>NT</td>
<td>90.2 ± 2.8%</td>
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mean ± SEM, *p < 0.02, **p < 0.01, ***p < 0.005.

test and analysis of variance. A level of p < 0.05 was taken as significant.

**RESULTS**

Figure 1 shows the example of the osmotic fragility of erythrocytes determined by the coil planet centrifuge method. Treatment of erythrocytes with Ca-ionophore A23187 and Ca in the bathing medium caused the reduction of the osmotic fragility dose-dependently on Ca-concentration. In preliminary study we found that A23187 alone or with other cations such as Mg⁺ did not reduce the osmotic fragility of erythrocytes. This finding provided evidence that the reduction of the osmotic fragility of erythrocytes shown in this experiment was due to the influx of Ca into the cells.

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Fig. 2. Relationship between Ca-induced changes of the erythrocyte osmotic fragility (OF) and plasma renin activity (PRA) in essential hypertension.

Fig. 3. Effects of Ca-antagonists (verapamil or diltiazem) or calmodulin-antagonist (trifluoperazine) on the changes of erythrocyte osmotic fragility by Ca-loading (A23187 0.9 μM, CaCl₂ 1.0 mM) in essential hypertension (ET) and normotensive subjects (NT).
Ca-antagonists or calmodulin-antagonist were preincubated with erythrocytes, and then Ca-loading was performed.

Alteration of the osmotic fragility of erythrocytes by Ca-loading in patients with essential hypertension.
Table I demonstrates the alteration of the osmotic fragility of erythrocytes by Ca-loading in essential hypertension and normotensive controls. The osmotic fragility in the Ca-free medium was somewhat higher in patients with essential hyper-
tension than in normotensive controls. When Ca was added into the medium, the osmotic fragility of erythrocytes was reduced both in the hypertensive patients and the normotensive controls. The absolute value of the osmotic fragility was lower in essential hypertension than in the control on the contrary to the Ca-free medium, and the changes of the osmotic fragility of erythrocytes by Ca-loading was significantly greater in hypertensive patients than in the controls (Table Ib).

Ca-induced changes of the osmotic fragility of erythrocytes and PRA

Figure 2 represents the relationship between Ca-induced changes of the osmotic fragility of erythrocytes (A23187 0.9 μM, CaCl₂ 1.0 mM) and PRA in essential hypertension. The alteration of the osmotic fragility by Ca-loading was inversely correlated with PRA in the patients with essential hypertension.

Effects of Ca-antagonists (verapamil, diltiazem) or calmodulin-antagonist ( trifluoperazine) on Ca-induced changes of the osmotic fragility of erythrocytes

In the presence of Ca-antagonists (verapamil or diltiazem) in the bathing medium, the reduction of the osmotic fragility by Ca-loading was inhibited, and the differences between the hypertensive and the normotensive groups were diminished or abolished by Ca-antagonists (Fig. 3).

Similar results were obtained in the presence of calmodulin-antagonist in the medium (Fig. 3).

DISCUSSION

It has been already reported that the alteration in osmotic fragility, size, shape and deformability of erythrocytes are regulated by the intracellular Ca levels. Some of these phenomena are considered to be due to the interactions of intracellular Ca to erythrocyte membrane proteins. Lake et al. observed that the changes in osmotic fragility induced by Ca-ionophore A23187 were correlated with net Ca uptake into the erythrocytes. So, it is likely that intracellular Ca contents could affect these physical properties of the erythrocyte membranes.

In addition, it has been proposed that erythrocyte membrane proteins, such as spectrin or actin, are contractile proteins and have similar characteristics to myosin and actin in the vascular smooth muscle cells, or cytoskeleton proteins in other cells.

On the basis of these previous findings, we examined the Ca-induced changes of the osmotic fragility of erythrocytes from patients with essential hypertension in order to investigate the membrane abnormalities of the Ca-handling in hypertension.

In this experiment, the osmotic fragility of erythrocytes in the Ca-free medium was greater in essential hypertension than that in normotensive controls, as previously reported. Treatment of the erythrocytes with Ca-ionophore A23187 and Ca caused reduction of the osmotic fragility on Ca-concentration in an almost dose-dependent manner both in essential hypertension and the normotensive controls. In addition, the alteration of the osmotic fragility of erythrocytes was greater in essential hypertension than in the normotension.

Ca-ionophore A23187 promotes Ca-entry into the cells, followed by increasing net Ca-influx and cytoplasmic Ca contents. A23187 also depolarizes cell membranes when Ca is added in the extracellular fluid.

Therefore, our results suggest that the Ca-influx across the cell membranes or interactions of Ca with erythrocyte membrane proteins could be enhanced in hypertension. If the same event occurs in other tissues, such as in vascular smooth muscles or sympathetic neurons, it could induce the increased vasoconstriction or exaggerated norepinephrine release from the nerve endings, since Ca has a major role in these responses.

Recently, some evidences have accumulated suggesting that the abnormality of the Ca-handling of the cells is a cause of hypertension. Postnov et al. reported that the Ca-binding ability of the erythrocyte membranes from essential hypertensive patients was reduced when compared with normotensive subjects, suggesting the increased intracellular free Ca in hypertension. Devynck et al. also indicated the reduced activity of the Ca-pump, and accelerated passive influx of Ca into the erythrocytes from spontaneously hypertensive rats. Indeed, Bruschi et al. already observed that intracellular cytoplasmic free Ca was increased in the platelets or lymphocytes of spontaneously hypertensive rats and essential hypertensive patients compared with their normotensive controls.

In clinical and experimental studies, it was demonstrated that Ca-antagonists, which are considered to block the Ca-influx across the membranes, reveal remarkable anti-hypertensive

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effects? Ishii et al. found that Ca-antagonists significantly reduced the blood pressure in hypertensive rats, but not in normotensive rats. Our previous reports have also showed that Ca-antagonists reduced the vascular responsiveness and noradrenaline overflow from the sympathetic nerve endings in the rat mesenteric vessels, and that the suppression was more prominent in spontaneously hypertensive rats than in normotensive controls. Therefore, it is strongly suggested that the increased Ca influx or elevated cytoplasmic free Ca might be responsible for the pathogenesis of hypertension at molecular levels, and that Ca-antagonists showed their hypotensive effects by suppressing increased Ca-sensitivity in hypertension.

In this study, the reduction of the osmotic fragility of erythrocytes by Ca-loading was inhibited in the presence of Ca-antagonists (verapamil or diltiazem) in the bathing medium, and the differences between the hypertensive and the normotensive groups were diminished or abolished by Ca-antagonists. These findings also provide evidence that Ca-influx was more suppressed in hypertension by Ca-antagonists than in the normotensive controls, and that the abnormalities of the Ca-handling of the cell membranes in hypertension could be corrected by Ca-antagonists. In addition, similar results were obtained by use of a calmodulin-antagonist, instead of Ca-antagonists. This observation proposed that the Ca-calmodulin system is also involved in the Ca-induced changes of the osmotic fragility of erythrocytes, and it might be activated in hypertension.

Müller et al. recently reported that Ca-antagonists were effective in low renin essential hypertensive patients, suggesting that Ca-sensitivity might be different according to the types of hypertension. The relationship between Ca and renin release is controversial. Many authors have reported that lowering extracellular Ca stimulates renin secretion, whereas raising Ca inhibits the secretion. Ca ionophore A23187 is also reported to inhibit renin release. These effects are exactly opposite to those on the other secretory systems, such as sympathetic noradrenaline release as described above. In the present study, it was demonstrated that Ca-induced changes of the osmotic fragility of erythrocytes were inversely correlated with PRA in essential hypertension. Although precise mechanisms of the renin release still remain to be identified, it is thought that Ca-sensitivity of the cell membranes could be increased in low renin essential hypertension.

From the rheological aspect, the increase in the stiffness of erythrocyte membranes after Ca-flux into the cells induces a loss of the deformability of erythrocytes, resulting in the increased viscosity of the blood and a disturbance of the microcirculation. In addition, elevated blood viscosity was observed in essential hypertension. Therefore, the increased intracellular Ca contents of the membranes might promote some rheological changes, and partly contribute to the pathogenesis of hypertension.

In summary, this study showed that the alteration of the osmotic fragility of erythrocytes by Ca-loading was greater in essential hypertension, especially in low renin hypertension than in the normotensive subjects. It might be due to a genetic abnormality of the Ca-handling of the cell membranes in hypertension. Furthermore, Ca-antagonists could reveal the anti-hypertensive actions by correcting this Ca-abnormality at cellular levels in hypertension.

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