**Alterations in Calcium-handling of Erythrocytes
in Spontaneously Hypertensive Rats**
**Calcium-induced Changes in the Osmotic Fragility
of Erythrocytes in Hypertension**

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SUMMARY

To investigate the sensitivity to calcium of erythrocytes in hypertension, changes in the osmotic fragility of erythrocytes following Ca-loading were observed. Washed erythrocytes were obtained from spontaneously hypertensive rats (SHR, Okamoto and Aoki) and age-matched normotensive Wistar Kyoto rats (WKY). Treatment of erythrocytes with Ca-ionophore A23187 and Ca in the medium caused a reduction in the osmotic fragility which correlated with the Ca-concentration. The degree of alteration in the osmotic fragility of erythrocytes was greater in SHR than in WKY. Oral administration of hydralazine to SHR significantly reduced the blood pressure. However, the alterations in the osmotic fragility of erythrocytes secondary to Ca-loading were not different between hydralazine-treated and untreated SHR.

In the presence of a Ca-antagonist (verapamil or diltiazem) in the medium, the reduction of the osmotic fragility of erythrocytes caused by Ca-loading was inhibited, and the differences between SHR and WKY were abolished by Ca-antagonists.

These results suggest that the greater changes in osmotic fragility of erythrocytes caused by Ca-loading in SHR could be due to a genetic abnormality of Ca-handling by the cell membranes, and that this abnormality might cause an increase in intracellular Ca, which contributes, in part, to the pathogenesis of hypertension.

Additional Indexing Words:

Spontaneously hypertensive rats Erythrocytes Osmotic fragility
Ca-handling Ca-antagonists Verapamil Diltiazem Hydralazine

RECENT evidence suggests that abnormalities of cellular ionic handling contribute to the pathogenesis of hypertension.¹⁾⁻³⁾ In particular,

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interest has been focused on the intracellular kinetics of Ca, since Ca has a major role in vasoconstriction, cardiac performance, neurotransmitter release from nerve endings and other important mechanisms of hypertension.^{4),5)}

The presence of widespread membrane defects could affect many organ systems, including circulating blood cells such as erythrocytes, lymphocytes or platelets. On the basis of this hypothesis, several studies have been performed using blood cells to clarify the cellular abnormalities in hypertension. Postnov et al reported that the Ca-binding ability of the erythrocyte membranes from patients with essential hypertension was reduced compared with that in normotensive subjects.⁶⁾ Bruschi et al observed that intracellular free Ca was increased in the platelets and lymphocytes of spontaneously hypertensive rats and essential hypertensive patients.^{7),8)}

An increase in the Ca-content of erythrocytes, on the other hand, can induce characteristic changes, such as reduced osmotic fragility, echinocyte formation or decreased deformability of the binding of intracellular Ca to the cytoskeleton proteins (spectrin, actin, ankyrin and other proteins) distributed in the erythrocyte membranes.^{9)–11)} These cytoskeleton proteins have characteristics in common with myosin and actin in the vascular smooth muscle cells and other contractile proteins in endocrine cells.¹²⁾ Thus, it is likely that the relationship between Ca and erythrocyte membranes is also present in other cells.

In the present study, to investigate the Ca-sensitivity of the cell membranes in hypertension, we examined alterations in the osmotic fragility of erythrocytes induced by Ca-loading in spontaneously hypertensive rats (SHR, Okamoto and Aoki) compared with age-matched normotensive Wistar Kyoto rats (WKY). In addition, the effects of Ca-antagonists on Ca-induced changes in osmotic fragility were studied in SHR.

MATERIALS AND METHODS

Fourteen to 16-week-old SHR and WKY were used. The systolic blood pressures, measured by the tail-cuff method (programmed electro-sphygmomanometer, PE-300, Narco Bio-System Inc., USA), were 183.0 ± 4.8 mmHg in SHR ($n=14$) and 129.8 ± 3.8 mmHg in WKY ($n=14$).

To investigate the effects of reduced blood pressure on the osmotic fragility of erythrocytes, hydralazine was administered to SHR ($n=15$, oral administration in drinking water, dose 5 mg/kg/day, $n=7$ and 10 mg/kg/day, $n=4$, in rats from 9–10 weeks old to 16–17 weeks old, untreated control SHR, $n=4$).

Determination of the osmotic fragility of erythrocytes by Ca-loading

Rats were anesthetized with pentobarbital (intraperitoneal injection, 40 mg/kg), and the abdominal cavity was opened. Blood was obtained from the inferior vena cava into heparinized tubes. Erythrocytes were washed 3 times with physiological saline, and incubated for 30 min in a buffered solution of 140 mM of NaCl and 20 mM of Tris-HCl at pH 7.4 and 37°C containing Ca-ionophore A23187 (0.9 μ M) and CaCl₂ (0–2.0 mM) (100 μ l of packed erythrocytes and 200 μ l of buffer). The osmotic fragility of erythrocytes was estimated by the coil planet centrifuge method.^{13)–15)} Incubated erythrocytes (10 μ l) were applied to the high osmotic end of the plastic coil (0.3 mm in diameter and 3 m in length) with an osmotic gradient from 150 milliosmol (mOsm) to 30 mOsm of NaCl. The coil was incubated at 37°C for 10 min, and centrifuged for 10 min at 1,600 rpm with 16 self-rotations / min by the coil planet centrifuge system. Erythrocytes were driven up through the coil in a gradient of NaCl from 150 mOsm to 30 mOsm, and hemolyzed where their membranes could not resist the disruptive force of the hypo-osmotic stress. The distribution of the hemoglobin from the ruptured erythrocytes was determined by a densitometer at 565 nm (DMU 33-C, Toyo Kagaku Sangyo Co. Ltd., Tokyo).

The osmotic fragility of erythrocytes was expressed as mOsm of NaCl at the hemolysis starting point (HSP), hemolysis maximum point (HMP) and hemolysis end point (HEP). It was sometimes difficult to determine HSP and HMP exactly, so we adopted HEP as an indicator of the osmotic fragility of erythrocytes.

Effects of a Ca-antagonist (verapamil or diltiazem) on Ca-induced changes in the osmotic fragility of erythrocytes

To investigate the effects of Ca-antagonists on Ca-induced changes in the osmotic fragility of erythrocytes, 100 μ l of packed erythrocytes were pre-incubated with 50 μ l of the buffer containing Ca-antagonists (140 mM NaCl, 20 mM Tris-HCl, pH 7.4, 37°C, with verapamil 5.0×10^{-4} – 1.0×10^{-3} M or diltiazem 5.0×10^{-4} M– 1.0×10^{-3} M). After a 3 hour incubation, Ca-ionophore A23187 (0.9 μ M) and CaCl₂ (1.0 mM) in 200 μ l of the buffer were added and erythrocyte Ca-loading was effected in the presence of a Ca-antagonist (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). The solution was then incubated for 30 min at 37°C and the osmotic fragility of the erythrocytes was measured by the coil planet centrifuge method as described above.

Values are expressed as mean $\pm$ SEM. Statistical significance was determined by the Student's t-test and analysis of variance. A level of $p < 0.05$

was considered to be significant.

Verapamil, diltiazem and A23187 were obtained from Eisai Co., Ltd., Tanabe Co., Ltd. and Hoechst Co., Ltd., respectively.

RESULTS

Changes in the osmotic fragility of erythrocytes induced by Ca-loading in SHR and WKY

Fig. 1 demonstrates the Ca-induced changes in the osmotic fragility of erythrocytes in SHR and WKY. The osmotic fragility was reduced in both groups dependent upon the Ca-concentration. In a preliminary study, we observed that A23187 alone or A23187 with other cations, such as Mg^{2+} , did not reduce the osmotic fragility of erythrocytes. This finding indicates

Fig. 1. Changes in the osmotic fragility of erythrocytes induced by Ca-loading (Ca-ionophore A23187 0.9 μ M and $CaCl_2$ 0–2.0 mM). The osmotic fragility of erythrocytes was evaluated by the coil planet centrifuge method. Arrows in the figure indicate HEP, as an indicator of the osmotic fragility. When erythrocytes are less fragile, the value of HEP is decreased.

Table I. Osmotic Fragility of Erythrocytes with Ca-loading in SHR and WKY

A23187	0.9 μ M	0.9 μ M	0.9 μ M	0.9 μ M
$CaCl_2$	(–)	0.5 mM	1.0 mM	2.0 mM
SHR (n=6)	72.6 $\pm$ 0.8	64.8 $\pm$ 0.9***	61.2 $\pm$ 1.2*	60.5 $\pm$ 1.5**
WKY (n=6)	75.5 $\pm$ 1.1	73.8 $\pm$ 0.7	66.7 $\pm$ 1.7	67.5 $\pm$ 0.9

Values are expressed as mOsm of NaCl at hemolysis end point (HEP) determined by the coil planet centrifuge method.

Statistical significances are indicated between SHR and WKY (mean $\pm$ SEM, * $p < 0.05$, ** $p < 0.005$, *** $p < 0.001$).

that the decrease in the osmotic fragility of erythrocytes was due to the influx of Ca^{2+} into the cells.

The osmotic fragility of erythrocytes in Ca-free medium was similar between SHR and WKY. When Ca was added to the medium with A23187, the values of osmotic fragility were lower in SHR than in WKY (Table I).

Effects of reduced blood pressure on Ca-induced changes in erythrocyte osmotic fragility in SHR

Oral administration of hydralazine to SHR significantly reduced the systolic blood pressures (untreated control SHR, 209.0 ± 5.0 mmHg, $n=4$; 5 mg/kg/day of hydralazine-treated SHR, 172.6 ± 7.1 mmHg, $n=7$, $p<0.01$; 10 mg/kg/day of hydralazine-treated SHR, 166.5 ± 3.4 mmHg, $n=4$, $p<0.01$). The changes in the osmotic fragility of erythrocytes induced by Ca-loading were, however, not different among these 3 groups (Fig. 2).

Effects of Ca-antagonists (verapamil or diltiazem) on Ca-induced changes in osmotic fragility of erythrocytes

In the presence of verapamil or diltiazem, the reduction in osmotic fragility of the erythrocytes by Ca-loading was inhibited, and the differences

Fig. 2. Effects of reduced blood pressure on the Ca-induced changes of erythrocyte osmotic fragility in SHR. Hydralazine was administered orally to SHR for 7 weeks (from 9–10 to 16–17 weeks old).

Fig. 3. Effects of Ca-antagonists (verapamil or diltiazem) on Ca-induced changes in osmotic fragility of SHR ($n=8$) and WKY ($n=8$).

between SHR and WKY were abolished by these Ca-antagonists (Fig. 3).

DISCUSSION

The present study shows that the treatment of erythrocytes with the Ca-ionophore A23187 and Ca causes a dose-dependent reduction in osmotic fragility in SHR and WKY, with the degree of alteration being significantly greater in SHR than in WKY.

It is generally accepted that A23187 promotes Ca-entry into the cells, resulting in increases in the Ca-influx as well as cytoplasmic Ca contents, and that A23187 depolarizes the cell membranes when Ca is present in the extracellular fluid.^{16),17)} Lake et al reported that the changes in osmotic fragility induced by A23187 correlated well with the net Ca-uptake into the erythrocytes.¹⁸⁾ In addition, intracellular Ca is thought to act on erythrocyte membrane proteins and alter the osmotic fragility of erythrocytes.¹¹⁾ Therefore, our results suggest that the Ca-influx across the erythrocyte membrane or the interaction between Ca and membrane proteins might be enhanced in SHR compared with normotensive WKY. Furthermore, the alterations in the osmotic fragility of erythrocytes induced by Ca-loading were

not different between hydralazine-treated and untreated SHR, which implies that this increased change in the osmotic fragility in SHR is due to a genetic abnormality of Ca-handling by the cell membranes, and not a consequence of high blood pressure. If the alterations observed in the SHR erythrocytes reflect a generalized abnormality of Ca-handling in other tissues, such as vascular smooth muscles, cardiac muscles or sympathetic neurons, it would enhance vasoconstriction, increase cardiac performance or exaggerate sympathetic nerve activity, since these responses are dependent upon the elevation of intracellular Ca concentrations. Recently, it has been proposed that Ca has a key role in the pathogenesis of hypertension, and that Ca-antagonists are useful antihypertensive agents that suppress transmembrane Ca-influx.^{19),20)} We reported that Ca-antagonists strongly inhibited vasoconstrictor responses and norepinephrine release from the nerve endings in SHR compared with age-matched WKY, suggesting that the Ca-dependency of cell membranes is marked in SHR.^{21),22)} Ishii et al observed that Ca-antagonists significantly reduced the blood pressure in hypertensive rats, but not in normotensive rats.²³⁾ Therefore, it is possible that the Ca-sensitivity of the cell membrane is increased in SHR and that the hypotensive action of Ca-antagonists corrects this abnormality of Ca-handling in hypertension. In this study, the reduction in the osmotic fragility of erythrocytes induced by Ca-loading was inhibited by verapamil and diltiazem, and the differences in the osmotic fragility induced by Ca-loading between SHR and WKY were diminished by these Ca-antagonists. These results provide evidence that Ca-antagonists are more effective in SHR than in WKY, suggesting that the Ca-influx or the intracellular Ca-kinetics were strongly suppressed by Ca-antagonists in SHR.

Erythrocyte membrane proteins, such as spectrin, actin or ankyrin appear to have some characteristics in common with contractile proteins in the vascular smooth muscle cells and cytoskeleton proteins in other tissues.^{11),12)} These proteins are partly regulated by the Ca-calmodulin complex in the cells and it is reported that Ca-antagonists also act on calmodulin.²⁴⁾ Therefore, the Ca-calmodulin system may also be involved in the Ca-induced changes in the osmotic fragility of erythrocytes, and may be activated in this model of hypertension.

In summary, the present study demonstrates that the changes in the osmotic fragility of erythrocytes induced by Ca-loading were greater in SHR than in WKY. This might be due to a genetic abnormality of Ca-handling by the cell membranes. The antihypertensive action of Ca-antagonists may correct the Ca-abnormality in hypertension.

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