Electron Spin Resonance Studies of Erythrocytes from Spontaneously Hypertensive Rats and Humans with Essential Hypertension

KAZUSHI TSUDA, HIDEO IWASHI, YOHSUKE MINATOGAWA, ICHIRO NISHIO, RYO KIDO, AND YOSHIKI MASUYAMA

SUMMARY The purpose of the present study was to investigate erythrocyte membrane abnormalities in hypertension by means of an electron spin resonance and spin-label technique. The erythrocytes from spontaneously hypertensive rats (SHR) and humans with untreated essential hypertension were examined and compared with their normotensive counterparts, and electron spin resonance spectra were obtained for a fatty spin-label agent (5-nitroxy stearate) incorporated into the erythrocyte membranes. The value of outer hyperfine splitting ($2T_o$) was significantly higher in erythrocytes of SHR and humans with essential hypertension than in erythrocytes of normotensive controls (at 37°C: SHR, 56.14 ± 0.51 gauss [G], n = 8; Wistar-Kyoto rats, 52.22 ± 0.86 G, n = 4, p < 0.01; humans with essential hypertension, 56.94 ± 0.27 G, n = 11; normotensive subjects, 55.44 ± 0.36 G, n = 8, p < 0.01). The order parameter ($S$) was also increased in the hypertensive rats and humans compared to their respective normotensive controls. When calcium was loaded to erythrocytes with calcium ionophore A23187 (0.9 μM) and CaCl₂ (1.0 mM), the parameters of the spectra were increased. These changes were more prominent in the hypertensive groups than in the normotensive controls. These results revealed that the erythrocyte membranes of the hypertensive subjects tolerated different spin motions than those of the normotensive controls in the electron spin resonance study and that membrane fluidity might be decreased in hypertension. Additionally, calcium loading to erythrocytes caused the reduction of membrane fluidity. Therefore, it is suggested that an abnormality of calcium handling at the cellular level might affect physical properties of the biomembranes in hypertension.

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KEY WORDS • essential hypertension • spontaneously hypertensive rats • erythrocytes • calcium • electron spin resonance • fatty acid spin labeling

MANY studies have shown that the biochemical and biophysical abnormalities of cell membranes are important factors in the etiology of hypertension in humans and animals.1-5 It seems likely that generalized abnormalities of the membranes could be involved not only in vascular smooth muscle cells but also in circulating blood cells. Erythrocytes are widely studied because they are a readily available source of cell membranes. Postnov et al. reported altered permeability of erythrocytes to sodium and potassium ions in spontaneously hypertensive rats (SHR).6 Friedman et al.7 and De Mendonca et al.8 observed abnormal sodium and potassium fluxes in erythrocytes from hypertensive rats. In addition to functional abnormalities of electrolyte handling, the structural or physical properties of erythrocytes from hypertensive rats have also been studied. Montheny-Garestier et al. reported about elevated viscosity in erythrocyte membranes from SHR.9 In another study, a fluorescent probe technique revealed diminished lateral diffusion in erythrocyte membranes from SHR.10

Recently, an electron spin resonance (ESR) and spin-label technique has been developed to elucidate the physicochemical properties of cell membranes and perturbations of these membranes by external...
agents.11-13 By means of a maleimide spin labeling, Gulak et al. reported the different ESR spectra between SHR and Wistar-Kyoto rats (WKY), suggesting that the protein structures in erythrocyte membranes might be altered in hypertension.14

To gain further insight into membrane abnormalities due to hypertension, we have examined a fatty acid spin-label signal from the erythrocytes of SHR and patients with essential hypertension and compared it to the signal from erythrocytes of normotensive controls. We also investigated calcium-related changes in ESR spectra of erythrocytes by use of calcium ionophore A23187.

Materials and Methods

Erythrocyte Preparation

Male SHR (Okamoto and Aoki strain) and WKY were obtained from Charles River (Kanagawa, Japan), and experiments were performed when the rats were 10 to 13 weeks old. The blood pressures, measured by the tail cuff method (programmed electrosphygmomanometer, Model PE-300; Narco Biosystem, Houston, TX, USA), were 181.4 ± 5 mm Hg (n = 9, mean ± SEM) in the SHR and 116.0 ± 2.5 mm Hg (n = 4) in the WKY. Body weights were 278.9 ± 4.5 g (n = 9) in the SHR and 295 ± 3.1 g (n = 4) in the WKY.

Eleven patients with essential hypertension (4 male, 7 female; aged 45.2 ± 2.5 years; blood pressures after 30-minute bed rest 160.5 ± 3.7/97.1 ± 2.6 mm Hg) were studied compared with normotensive subjects (5 male, 3 female; aged 41.8 ± 2.6 years; blood pressure 130.7 ± 2.8/75.0 ± 3.3 mm Hg). All the hypertensive patients had stage I or II hypertension (World Health Organization classification) and they had no medication for at least 2 weeks prior to the study. They had no other diseases, such as hematologic or hepatic disorders. The levels of serum protein, cholesterol, triglycerides, sodium, and potassium, and other routine laboratory findings were about the same in the hypertensive and the normotensive controls.

The rats were anesthetized with pentobarbital (40 mg/kg, intraperitoneal injection) and the abdominal cavity was opened. Heparinized blood was obtained from the inferior vena cava of each rat. For the human subjects, blood sampling was performed by venipuncture after at least 30 minutes of bed rest. After plasma and buffy coat were carefully removed by centrifugation, erythrocytes were washed three times in physiological saline. The cells were resuspended in the isotonic buffer (140 mM sodium chloride, 20 mM Tris HCl, pH 7.4) at a hematocrit of 50%.

Spin Labeling and Electron Spin Resonance

The erythrocyte suspension was preincubated for 30 minutes at 37°C, and then spin labeling was performed.15 A fatty acid spin label, 5-nitroxy stearate (5-NS; 2,3-carboxypropyl-4,4-dimethyl-2-tridelyl-3-oxazolidinolxyloxy) was purchased from Syva Associates (Palo Alto, CA, USA) and kept at a stock solution of 0.01 M in 100% ethanol. The agent was diluted in NaCl-Tris buffer before the experiment (5 × 10⁻⁵ M).

Two hundred microliters of the buffer containing spin label was added to 400 µl of erythrocytes (concentration of spin-label agent vs erythrocyte membrane protein, about 0.8 µg/mg), and the solution was incubated for 2 hours at 37°C with gentle shaking.

ESR was measured using an ESR spectrometer (JES-FE2XG, Nihon Denshi, Tokyo, Japan) with a microwave control unit (ES-SCX, Nihon Denshi). The temperature was controlled by an ES-DVT1 control unit (Nihon Denshi), and its accuracy was within ±0.5°C.

After being washed in isotonic NaCl-Tris buffer, erythrocytes were packed in a quartz ESR flat cell (ES-LC11, Nihon Denshi). The microwave power was 5 mW, and the modulation frequency was 100 kHz, with an amplitude of 2.0 gauss (G). The recorder scan width was 3280 ± 50 G, and the sweep time was 8 minutes. The receiver gain was 4.0 × 10³ to 7.9 × 10³, with a response time of 1.0 second.

Effects of A23187 on Electron Spin Resonance Spectra

Washed erythrocytes were incubated for 30 minutes at 37°C in NaCl-Tris buffer containing 0.9 µM of calcium ionophore A23187 (Calbiochem-Behring, Hoechst, and 1.0 mM of CaCl₂ (hematocrit 50%). Then 200 µl of NaCl-Tris buffer containing spin label was added to 400 µl of erythrocytes. After incubation at 37°C for 2 hours, ESR spectra were obtained by the procedures described above.

All values are expressed as means ± SEM. Statistical significance was determined by an unpaired or paired Student's t test. A value of p < 0.05 was considered to be significant.

Results

Fatty acid spin-label agents are considered to be anchored at the lipid-aqueous interface of the cell membranes by their carboxyl ends, whereas the nitroxide groups move rapidly through a restricted angle around the point of attachment.16, 17 Therefore, the ESR spectra of the fatty acid spin label are used to detect an alteration of the freedom of the motion in biological membranes and provide an indication of membrane fluidity.15, 17

Figure 1 shows a typical ESR spectrum for 5-NS in erythrocytes from a normotensive subject. Outer and inner hyperfine splitting (2T1, 2T1) in gauss (G) were evaluated in each ESR spectrum, and order parameter (S) was calculated from 2T1 and 2T1 values as follows.5, 18

\[ S = \frac{T_1 - T_{1s}}{T_{ss} - T_{1s}} \times \frac{a_e}{a_o} \]

where \( T_{ss}, T_{1s} = \) hyperfine constant, and \( a_e/a_o = \) isotropic coupling constant. The greater values of 2T1 and S are associated with the lesser freedom of motion of the spin label in the biomembrane bilayers, indicating decreased membrane fluidity.

Table 1 represents the values of ESR spectral parameters of hypertensive groups and their normotensive
controls. The ESR spectra were taken at 30°C, 37°C, and 37°C. As shown, $2T_J$ and $S$ were significantly higher in SHR than in normotensive WKY at both 30 and 37°C. Similarly, the ESR parameters of erythrocytes from patients with essential hypertension were increased compared to those from normotensive control subjects.

Figure 2 demonstrates the effects of calcium loading (CaCl$_2$ with calcium ionophore A23187) on ESR spectra of erythrocytes from SHR and WKY. The order parameter ($S$) was significantly increased in erythrocytes from SHR when calcium loading was performed ($S$ value at 30°C, 0.711 ± 0.003–0.731 ± 0.009; $n = 6$; $p < 0.05$ by a paired $t$ test). Increases for erythrocytes from WKY were less significant. The $S$ value in the presence of calcium and calcium ionophore was also higher for SHR than for WKY (SHR, 0.731 ± 0.009; WKY, 0.655 ± 0.003; 30°C; $p < 0.05$).

Figure 3 shows the changes of ESR spectra of erythrocytes from human subjects with essential hypertension. Like the $S$ value for SHR, the $S$ value for humans with essential hypertension was significantly increased when calcium loading was performed, and it was higher than the $S$ value in controls ($S$ value of 0.721 ± 0.013 in essential hypertension vs 0.670 ± 0.003 in normotensive controls, $p < 0.05$).

**Discussion**

In recent years, ESR and spin-label techniques have been widely used to obtain information about the composition, structure, and function of biomembranes. Spin-label agent 5-NS can be inserted into lipid membranes, and analysis of the signals from 5-NS in the membranes provides an indication of membrane fluidity.

In the present study, we showed that hyperfine splitting ($2T_J$) and order parameter ($S$) in the erythrocytes from SHR and human subjects with essential hypertension were greater than those from normotensive controls, suggesting that erythrocyte membrane fluidity is decreased in hypertension.

It is well known that membrane fluidity is related to the microviscosity of the membrane and its lipid composition, water content, divalent cation content, or

**Table 1.** Electron Spin Resonance Spectrum Parameters for 5-Nitroxy Stearate in Hypertensive and Normotensive Subjects

<table>
<thead>
<tr>
<th>Subjects</th>
<th>$2T_J$ (G)</th>
<th>$S$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>30°C</td>
<td>37°C</td>
</tr>
<tr>
<td>Rats</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SHR</td>
<td>57.11 ± 0.25* ($n = 9$)</td>
<td>56.14 ± 0.51† ($n = 8$)</td>
</tr>
<tr>
<td>WKY</td>
<td>53.88 ± 0.40 ($n = 4$)</td>
<td>52.22 ± 0.86 ($n = 4$)</td>
</tr>
<tr>
<td>Humans</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EH</td>
<td>—</td>
<td>56.94 ± 0.27† ($n = 11$)</td>
</tr>
<tr>
<td>NT</td>
<td>—</td>
<td>55.44 ± 0.36 ($n = 8$)</td>
</tr>
</tbody>
</table>

Values are expressed as means ± SEM.

$p < 0.005$, †$p < 0.01$, compared with normotensive controls.
protein-lipid interactions. Additionally, changes in ionic transport, receptor and enzymatic functions, or physical properties, such as deformability of the membrane, are thought to be influenced by an alteration of membrane fluidity. Wiley and Cooper reported that the changes in the cholesterol-phospholipid ratio of erythrocyte membranes could affect membrane fluidity as well as cotransport of sodium and potassium, membrane permeability to several substances, or the diffusion of substances across the membrane. Yamori et al. observed that the cholesterol content of erythrocyte membranes from stroke-prone SHR was less than the cholesterol content of membranes from WKY. Boriskina et al. reported that the phosphatidyl inositol content in erythrocytes from SHR was decreased. Naftilan and co-workers studied the fluidity and lipid composition of platelet membranes from patients with essential hypertension and observed that these membranes were less fluid (as determined by fluorescence) and contained less linoleic acid than membranes of platelets from normotensive controls. Yeagle showed that erythrocyte lipids markedly affected $\text{Na}^+,\text{K}^+-\text{ATPase}$ activity. Therefore, it is possible that changes in the lipid components of erythrocyte membranes cause decreased membrane fluidity in hypertension, as well as abnormalities of ionic transport across the membranes.

It is widely accepted that intracellular calcium levels can regulate the size, shape, or other physical properties of erythrocytes, such as deformability and osmotic pressure.

**Figure 2.** Effects of calcium loading on electron spin resonance spectra of erythrocytes from spontaneously hypertensive rats (SHR) and normotensive Wistar-Kyoto rats (WKY). Calcium loading to erythrocytes was performed with calcium ionophore A23187 (0.9 μM) and CaCl$_2$ (1.0 mM) in the incubation medium. Data in the upper graph were obtained at 30°C, and data in the lower graph were obtained at 37°C. The effects of calcium loading were evaluated as changes in order parameter (S). Numbers above the graph bars indicate number of animals.

**Figure 3.** Effects of calcium loading on electron spin resonance spectra of erythrocytes from humans with essential hypertension (EH) and normotensive controls (NT). The measurement was performed at 37°C. The procedure for calcium loading was the same as that described in Figure 2. Numbers above the graph bars indicate number of subjects.
fragility. These phenomena are considered to be related to the interactions between intracellular calcium and the membrane. Calcium ionophore A23187 is known to promote calcium entry into the cells, which is followed by an increase in net calcium influx and cytoplasmic calcium. Lake et al. reported that calcium ionophore A23187 induced net calcium uptake by erythrocytes that was concomitant with changes in osmotic fragility. Therefore, it is likely that an increase in intracellular calcium content could affect some physical properties of the erythrocyte membrane.

On the basis of these previous findings, we investigated the calcium-induced changes in membrane fluidity of erythrocytes from SHR and humans with essential hypertension in comparison with their respective normotensive controls. Treatment of the erythrocytes with calcium ionophore A23187 and calcium caused a decrease in membrane fluidity (27° and S were increased), and the decrease was more prominent in SHR and humans with essential hypertension than in controls. Previously, we reported that treatment with A23187 and calcium reduced the osmotic fragility of erythrocytes, suggesting an increase in membrane rigidity. We also found that the alteration of osmotic fragility was greater in subjects with hypertension than in normotensive controls. Although the precise mechanisms explaining these phenomena are still unknown, our results indicate that calcium influx across the cell membranes or interactions between calcium and erythrocyte membranes (including protein-lipid interactions) might be enhanced by hypertension. If the same phenomenon occurs in the cells of other tissues, such as vascular smooth muscles, it would cause vascular resistance to increase because calcium has a major role in vasoconstriction, and membrane fluidity might be inversely correlated with membrane stiffness.

Recently, many other spin-label agents have been developed, such as phospholipid and steroid spin-label agents. The use of these agents might provide more data about the effects of hypertension on biomembranes.

In summary, ESR studies with a fatty acid spin-label agent revealed that the erythrocyte membranes of SHR and human subjects with essential hypertension resulted in different spin motions from those of normotensive controls, and that the membrane fluidity of erythrocytes was decreased in hypertension. Calcium loading to erythrocytes caused reduced membrane fluidity, which was more prominent in erythrocytes from SHR and humans with essential hypertension than in their respective normotensive controls. These results suggest that an abnormality of calcium handling at cellular levels might affect the physical properties of biomembranes in subjects with hypertension.

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