Role of Insulin in the Regulation of Membrane Fluidity of Erythrocytes in Essential Hypertension: An Electron Paramagnetic Resonance Investigation

Kazushi Tsuda, Yukiko Kinoshita, Ichiro Nishio, and Yoshiaki Masuyama

In the present study, to determine a possible role of insulin in the regulation of membrane functions, we have examined the effects of insulin on the membrane fluidity of erythrocytes in patients with essential hypertension and normotensive subjects. Membrane fluidity of erythrocytes obtained from hypertensive and normotensive subjects were evaluated by means of an electron paramagnetic resonance (EPR) and a spin-labeling method. In an in vitro study, insulin increased the order parameter (S for 5-nitroxide stearate) and the peak height ratio (ho/h-1 for 16 nitroxide stearate) in the EPR spectra of erythrocyte membranes, which indicated that insulin decreased the membrane fluidity of erythrocytes. The effects of insulin on the membrane fluidity were potentiated in the presence of extracellular Ca\(^{2+}\), and in contrast, were antagonized by the Ca\(^{2+}\) channel blocker diltiazem. Furthermore, the effects of insulin alone and in combination with Ca\(^{2+}\) on the membrane fluidity were reduced in the erythrocytes from hypertensive subjects compared with the erythrocytes from normotensive controls. The high concentrations of glucose alone produced no significant effects on the membrane fluidity of erythrocytes. These results demonstrated that insulin might actively participate in the regulation of membrane fluidity of erythrocytes, which might be mediated by the intracellular Ca\(^{2+}\) kinetics.


KEY WORDS: Insulin, membrane fluidity, erythrocytes, electron paramagnetic resonance, essential hypertension, calcium.

Many studies have provided evidence that insulin resistance and associated hyperinsulinemia are causally related to hypertension.\(^1,2\) It was shown that the fasting and postprandial insulin levels were significantly higher in patients with essential hypertension than in normotensive subjects.\(^3,4\) It was also demonstrated that insulin influenced several transmembrane ionic transport systems, including the Ca\(^{2+}\)–ATPase, the Na\(^+\)–Ca\(^{2+}\) exchange systems, the Na\(^+,\) K\(^+\)–ATPase, and the Na\(^+\), H\(^+\)–transport systems.\(^5\)–\(^9\)

It has been proposed that cell membrane abnormal-

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ities are an etiologic factor in hypertension. An electron paramagnetic resonance (EPR) and spin-labeling method have been developed to elucidate the membrane fluidity and perturbations of the membrane function by external agents. The membrane fluidity is a physicochemical feature of biomembranes, and has an important role in modulating cell functions such as rheologic behavior and membrane microviscosity. Using the EPR method our previous studies showed that the membrane fluidity of erythrocytes was significantly lower in both spontaneously hypertensive rats and patients with essential hypertension than in the normotensive controls. However, the mechanisms responsible for this phenomenon are still unclear. In the present study, we have focused on the role of insulin in the regulation of membrane fluidity of erythrocytes in patients with essential hypertension and normotensive subjects by means of the EPR method.

METHODS

Subjects To investigate the effects of insulin on the membrane fluidity in vitro, the erythrocytes were obtained from the volunteers who were divided into normotensive and hypertensive groups (normotensives, n = 41, 36 men, 5 women; aged 57 ± 2 years; blood pressure, 119.3 ± 2.3/69.2 ± 1.5 mm Hg; heart rate, 70.0 ± 6.7 beats/min; body mass index, 22.8 ± 0.7 kg/m²; fasting blood sugar, 5.6 ± 0.2 mmol/L; hypertensives, n = 22, 20 men, 2 women, aged 59 ± 2 years; blood pressure, 157.3 ± 2.6/90.0 ± 2.2 mm Hg; heart rate, 72.3 ± 1.7 beats/min; body mass index, 23.0 ± 0.4 kg/m²; fasting blood sugar, 5.9 ± 0.2 mmol/L). Consent was obtained from all volunteers after they were explained about the nature and objective of the study. The hypertensive group consisted of borderline and mild hypertensive patients who had taken no medication for at least 2 weeks before the study. They had no other diseases such as hemato logic or hepatic disorders. Blood sampling (10 mL) was performed by venipuncture after a minimum of 30 min of bed rest while fasting. Heparin (100 µL) was used as an anticoagulant agent. After plasma and Buffy coat were carefully removed by centrifugation at 155 g for 10 min at 4°C, washed erythrocytes were resuspended in the isotonic buffer (140 mmol/L NaCl, 20 mmol/L Tris-HCl, pH 7.4) at a hematocrit of 50%. Effects of Insulin Alone and in Combination With Calcium and the Calcium Channel Blocker on Membrane Fluidity of Erythrocytes In Vitro The erythrocyte suspension (erythrocytes 100 µL and Tris-HCl buffer 100 µL; 200 µL total) was incubated for 2 h at 37°C in the NaCl-Tris buffer (100 µL) containing insulin (6 × 10⁻¹¹ to 6 × 10⁻⁸ mol/L) alone and in combination with 1.0 mmol/L of CaCl₂. To determine the effects of the Ca²⁺ channel blocker, erythrocytes (100 µL) were pretreated with the same volume of Tris-HCl solution containing diltiazem (1 × 10⁻⁵ mol/L for 30 min at 37°C) before the application of insulin (6 × 10⁻¹⁰ mol/L) and CaCl₂ (1.0 mmol/L). Then, the solution containing the spin-label agents (5-nitroxide stearate [5-NS] and 16-nitroxide stearate [16-NS], 5 × 10⁻⁵ mol/L in 100 µL of Tris-HCl buffer) was added to the erythrocyte suspension. After 2 h of incubation at 37°C, the EPR spectra were obtained.

Effects of Glucose on Membrane Fluidity of Erythrocytes In Vitro To study the effects of glucose on the membrane fluidity, 100 µL of glucose (10 and 20 mmol/L) or same concentration of urea (as a control) was applied to 200 µL of erythrocyte suspension. After incubation of the mixed solution for 2 h at 37°C, the spin-labeling agent-containing solution (100 µL) was added, and the EPR measurements were performed.

EPR Measurements The EPR measurements were performed using an EPR spectrometer (model Jeol JES-FF2XG, Nihon Denshi, Tokyo, Japan) with a microwave unit (model Jeol ES-SCXA, Nihon Denshi) as described previously. The microwave power was 5 mW, and the modulation frequency was 100 KHz with a modulation amplitude of 2.0 gauss (G). The temperature of the measurement was controlled at 30°C. The receiver scan width was 3280 ± 50 G with a sweep time of 8 min, and receiver gain was 4.0 × 10³ to 7.9 × 10³ with a response time of 1.0 sec. For indicators of membrane fluidity, we have evaluated the values of outer and inner hyperfine splitting (2T₁ and 2T₂ in G, respectively) in the EPR spectra for 5-NS and calculated the order parameter (S) from 2T₁ and 2T₂. In the EPR spectra for 16-NS, we used the peak height ratio (ho/h₁) value for an index of the membrane fluidity. The greater the values of the order parameter (S) and the peak height ratio (ho/h₁), the lesser the freedom of motion of the spin labels in the biomembrane bilayers and the membrane fluidity. Drugs The spin-label agents 5-NS and 16-NS were purchased from Aldrich Co., Ltd. (Milwaukee, WI). Insulin (human) was obtained from Shionogi Co., Ltd. (Osaka, Japan). All other drugs used were standard laboratory reagents of analytical grade.

Statistics Values are expressed as means ± SEM. Differences between the means of the drug treatment and their corresponding controls were tested with a one-way analysis of variance (ANOVA). To compare the means of the different study groups, Student’s paired t test was used. The differences were confirmed by Wilcoxon signed rank-sum test. The differences between hypertensive and normotensive subjects were analyzed with the two-way ANOVA followed...
by the Mann-Whitney U test. A $P$ value less than 0.05 was accepted as the level of significance.

**RESULTS**

Effects of Insulin Alone and in Combination With Calcium on Membrane Fluidity of Erythrocytes in Normotensive Subjects  Figure 1 showed the effects of exogenously applied insulin on the membrane fluidity of erythrocytes obtained from normotensive volunteers in vitro. Insulin ($6 \times 10^{-11}$ to $6 \times 10^{-8}$ mol/L) significantly increased the values of $S$ and $ho/h-1$ of the EPR spectra in a dose-related fashion. In the separate experiment, insulin in combination with $Ca^{2+}$ (1.0 mmol/L) increased the values of $S$ and $ho/h-1$ to a greater extent than the same concentrations of insulin alone, although $Ca^{2+}$ showed no significant effects on the membrane fluidity by itself (Table 1). The finding indicated that insulin significantly decreased the membrane fluidity of erythrocytes, and that the effect was more pronounced in the presence of extracellular $Ca^{2+}$. Furthermore, it was shown that the effects of insulin alone and in combination with extracellular $Ca^{2+}$ on the values of $S$ and $ho/h-1$ were antagonized by the $Ca^{2+}$ channel blocker diltiazem (Figure 2).

Effects of Insulin Alone and in Combination With Calcium on Membrane Fluidity of Erythrocytes in Hypertensive Subjects  Table 1 also shows the effects of insulin alone and in combination with $Ca^{2+}$ on the membrane fluidity of erythrocytes in hypertensive subjects. The basal value of the order parameter ($S$) before exposure to insulin was $0.733 \pm 0.004$ (n = 10) in hypertensive subjects (EH) and $0.702 \pm 0.005$ in normotensive subjects (NT).

**TABLE 1. EFFECTS OF INSULIN IN COMBINATION WITH $Ca^{2+}$ ON MEMBRANE FLUIDITY OF ERYTHROCYTES**

<table>
<thead>
<tr>
<th>Index for Membrane Fluidity</th>
<th>Control</th>
<th>$Ca^{2+}$ (1.0 mmol/L)</th>
<th>Insulin ($6 \times 10^{-10}$ mol/L)</th>
<th>Insulin ($6 \times 10^{-8}$ mol/L)</th>
<th>Insulin ($6 \times 10^{-10}$ mol/L) + $Ca^{2+}$</th>
<th>Insulin ($6 \times 10^{-8}$ mol/L) + $Ca^{2+}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Order parameter ($S$)</td>
<td>NT (n = 10)</td>
<td>0.702 ± 0.005</td>
<td>0.704 ± 0.005</td>
<td>0.717 ± 0.005*</td>
<td>0.722 ± 0.006*</td>
<td>0.731 ± 0.005*</td>
</tr>
<tr>
<td></td>
<td>EH (n = 10)</td>
<td>0.733 ± 0.004†</td>
<td>0.731 ± 0.004†</td>
<td>0.739 ± 0.004†</td>
<td>0.749 ± 0.003‡</td>
<td>0.746 ± 0.004†</td>
</tr>
<tr>
<td>Peak height ratio (ho/h-1)</td>
<td>NT (n = 10)</td>
<td>4.80 ± 0.09</td>
<td>4.85 ± 0.09</td>
<td>4.99 ± 0.07*</td>
<td>5.00 ± 0.04*</td>
<td>5.21 ± 0.04†</td>
</tr>
<tr>
<td></td>
<td>EH (n = 10)</td>
<td>5.13 ± 0.05‡</td>
<td>5.15 ± 0.06‡</td>
<td>5.25 ± 0.05‡</td>
<td>5.30 ± 0.05†</td>
<td>5.39 ± 0.05‡</td>
</tr>
</tbody>
</table>

Values are means ± SEM.

* $P < .05$ compared with the control value; † $P < .05$ compared with same concentrations of insulin alone; ‡ $P < .05$ between normotensive subjects (NT) and hypertensive subjects (EH).

Insulin ($6 \times 10^{-10}$ mol/L to $6 \times 10^{-8}$ mol/L) and $CaCl_2$ (1.0 mmol/L) were added to the erythrocyte suspension in vitro, and the spin labeling was performed as described in the Method section.
(n = 10) in normotensive subjects (NT) (P < .05). The peak height ratio (ho/h-1) was also greater in hypertensive subjects than in normotensive subjects (EH 5.13 ± 0.05, n = 10; NT 4.80 ± 0.09, n = 10; P < .05). The percent changes in membrane fluidity in the presence of insulin and Ca2+ were shown in Figure 3. The effect of insulin on the order parameter (S) (percent increase in S) was attenuated in hypertensive subjects compared with the normotensive subjects (F value between EH and NT = 19.226, P = .0001 by two-way ANOVA) (Figure 3). The increase in the peak height ratio (ho/h-1) also tended to be smaller in hypertensive subjects than in the normotensive subjects (F value between EH and NT = 4.184, P = .0432 by two-way ANOVA).

Effects of Glucose on Membrane Fluidity of Erythrocytes in Hypertensive and Normotensive Subjects

Figure 4 demonstrates the effects of glucose (10 and 20 mmol/L) on the values of S and ho/h-1 of the erythrocyte EPR spectra. There were no significant differences in the membrane fluidity between glucose-treated erythrocytes and the same concentration of urea (as a control)-treated erythrocytes in both hypertensive and normotensive subjects. The results indicated that these concentrations of glucose alone did not produce any significant effects on the membrane fluidity of erythrocytes.

DISCUSSION

In the present study we determined the role of insulin in the regulation of membrane fluidity of erythrocytes in patients with essential hypertension and normotensive subjects by means of the EPR and spin-labeling method. In an in vitro study, we demonstrated that insulin increased the values of the order parameter (S) and the peak height ratio (ho/h-1) of the EPR spectra. The result indicates that insulin lowered the membrane fluidity of erythrocytes, and further suggests that hyperinsulinemia could be a crucial determinant factor in regulating cell function.

The precise mechanisms underlying the insulin effects on membrane fluidity are still uncertain. There has been much evidence showing the importance of Ca2+ in the mechanisms of insulin action in several tissues.23–25 The results of the present study showed that insulin in combination with Ca2+ lowered the membrane fluidity of erythrocytes to a greater extent than insulin alone. The mean fluidity values in normo-
tensive subjects (shown in Figure 1) and in normotensive subjects (Table 1) in the absence of insulin were somewhat different. It has been shown that many factors, such as hematologic disorders, changes in electrolyte balance (in particularly sodium and Ca$^{2+}$), lipid levels, and other hormonal agents, might influence the membrane fluidity, which could partially explain that there were some differences in the fluidity values between the groups. With regard to the interactions between Ca$^{2+}$ and the membrane fluidity, it was observed that Ca$^{2+}$ strongly decreased the membrane fluidity of erythrocytes and other cells. We also reported that the treatment of erythrocytes with Ca$^{2+}$ and the Ca$^{2+}$ ionophore A23187 reduced the erythrocyte membrane fluidity. It is likely that the insulin-evoked decrease in the membrane fluidity may be partially mediated by the increased intracellular Ca$^{2+}$ concentration. By using the nuclear magnetic resonance method, Barbagallo et al demonstrated that insulin significantly elevated the cytosolic-free Ca$^{2+}$ level of human erythrocytes in a dose- and time-dependent manner. Recent studies have revealed that erythrocytes may contain a carrier similar to the slow Ca$^{2+}$ channels. David-Dufilho et al demonstrated that nifedipine reduced Ca$^{2+}$ influx in human erythrocyte and participated in the control of intracellular Ca$^{2+}$. Oonishi et al observed that Ca$^{2+}$ channel blockers inhibited the impairment of filterability of human erythrocytes under mechanical stress, and proposed that membrane properties regulating red cell deformability were affected by the signal transduction system, including Ca$^{2+}$-dependent pathways that might be sensitive to the L-type Ca$^{2+}$ channel blockers. Our present results showed that the changes in membrane fluidity of erythrocytes induced by insulin and Ca$^{2+}$ were inhibited by diltiazem. It would be possible that the insulin-evoked decrease in the erythrocyte membrane fluidity was due to the Ca$^{2+}$ influx from the extracellular space followed by an increase in the intracellular Ca$^{2+}$ concentration. One additional possible mechanism is that insulin stimulated directly the protein kinase C-mediated mechanisms and modulated the membrane fluidity of erythrocytes because the protein kinase C activity in the cells was activated by insulin, whereas the activity was inhibited by diltiazem. It might be possible that a part of the insulin effect was mediated by the intracellular Ca$^{2+}$ movement.

Perikanova et al observed the relationship between insulin and erythrocyte membrane composition in healthy men, and reported that fatty acid pattern of membrane total phospholipid may be linked to the properties of insulin receptors. Further studies should be conducted to assess more thoroughly the relationship between insulin and membrane functional and structural characteristics of erythrocytes.

In this study, it was shown that the effects of insulin alone and in combination with Ca$^{2+}$ on the membrane fluidity, particularly on the value of S, were reduced in the erythrocytes from hypertensive subjects compared with the erythrocytes from normotensive controls. It has been reported that the number of insulin receptors on the cell membranes was significantly decreased in hypertensives compared with the normotensive controls. With chronic exposure to high levels of insulin, there might be a down-regulation of the insulin receptors on the membranes in hypertension, although 2 weeks off medication may not be sufficient to negate the possible influence of antihypertensive drugs on membrane functions. Barbagallo et al reported that intracellular Ca$^{2+}$ actions of insulin were dependent on basal intracellular Ca$^{2+}$ and Mg$^{2+}$, and that the higher the initial Ca$^{2+}$ level, the less the effect of insulin, which might be consistent with our present result. It would be possible that an abnormality in the cell function such as ionic transport systems or membranous Ca$^{2+}$ handling, rather than the insulin action, might produce the alterations in membrane properties in hypertension. However, it does not diminish the importance of the role of insulin regulating membrane fluidity. It seems likely that a primary ionic defect in combination with Ca$^{2+}$-related action of insulin might synergistically elevate cytosolic Ca$^{2+}$ and further decrease membrane fluidity of erythrocytes in essential hypertension.

It has been shown that an elevated cytosolic Ca$^{2+}$ induced the phosphorylation of cytoskeletal proteins such as spectrin of erythrocyte membranes and further produced the protein–lipid interaction, which might decrease the membrane fluidity of erythrocytes. Because membrane fluidity is a reciprocal value of membrane microviscosity, the decreased membrane fluidity of erythrocytes might cause a disturbance in blood rheologic behavior and microcirculation, which could, at least in part, contribute to the pathophysiology of hypertension.

Our present study also showed that glucose produced no significant effects on membrane fluidity. Resnick and colleagues have reported that glucose itself elevated cytosolic Ca$^{2+}$ in a dose- and time-dependent manner. The reason for the discrepancy between the results is still uncertain. Williams and Schrier reported that elevated extracellular glucose depressed basal and voltage-sensitive Ca$^{2+}$ uptake in vascular smooth muscle cells. In addition, they demonstrated the glucose-induced down-regulation of angiotensin II and arginine vasopressin receptors on the membranes. It might be possible that the effect of glucose-induced increase in the intracellular Ca$^{2+}$ content could be counteracted by other glucose-induced membrane alterations. Further studies are necessary to assess more thoroughly the role of glu-
cose in the regulation of $Ca^{2+}$ transport across the cell membranes.

In summary, the results of the present study suggest that insulin may actively participate in the regulation of membrane fluidity of erythrocytes, a part of which could be mediated by the modulation of the intracellular $Ca^{2+}$ kinetics. Although further studies should be conducted to assess the precise mechanisms underlying the insulin action on the membrane fluidity and their participation in blood pressure control, hyperinsulinemia may constitute a crucial effect on the physiological properties of the cell membranes of human erythrocytes.

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**REFERENCES**


