Nitric Oxide Is a Determinant of Membrane Fluidity of Erythrocytes in Postmenopausal Women: An Electron Paramagnetic Resonance Investigation

Kazushi Tsuda, Yukiko Kinoshita-Shimamoto, Keizo Kimura, and Ichiro Nishio

In the present study, to determine a possible role of nitric oxide (NO) in the regulation of membrane functions, we examined the relationship between plasma NO level and membrane fluidity of erythrocytes in postmenopausal women. We evaluated the membrane fluidity of erythrocytes obtained from hypertensive and normotensive postmenopausal women by means of an electron paramagnetic resonance (EPR) and spin labeling method. The EPR study revealed that the order parameter (S) for 5-nitroxide stearate in erythrocyte membranes was significantly greater in hypertensive postmenopausal women than in normotensive postmenopausal women. The finding indicated that the membrane fluidity of erythrocytes was decreased in hypertensive postmenopausal women compared with normotensive postmenopausal women. The plasma level of the NO metabolites (nitrite and nitrate) while fasting was significantly lower in hypertensive postmenopausal women than in normotensive postmenopausal women. In addition, the order parameter (S) in the EPR spectra of erythrocyte membranes was inversely correlated with the plasma NO metabolite level, which indicated that the lower membrane fluidity of erythrocytes was associated with the lower plasma NO level in postmenopausal women. These results are consistent with the hypothesis that NO may have a crucial role in the regulation of membrane fluidity of erythrocytes in postmenopausal women. Am J Hypertens 2003;16:244–248 © 2003 American Journal of Hypertension, Ltd.

Key Words: Postmenopausal women, hypertension, nitric oxide, membrane fluidity, erythrocytes, electron paramagnetic resonance.

Many studies have focused on the cardioprotective effects attributable to nitric oxide (NO) and have shown that hypertension and other circulatory disorders may be associated with insufficient NO production and availability.1–3 It is well known that NO decomposes rapidly in biologic solutions with nitrite (NO2−) and nitrate (NO3−).4 These stable compounds can be analyzed in serum, and the level of the NO metabolites may be an index of the NO activity in vivo.5 It was demonstrated that plasma level of nitrite and nitrate was significantly lower in patients with essential hypertension than in normotensive subjects,6 which indicated that the production and availability of NO might be disturbed in hypertension.

In postmenopausal women, the content of ovarian hormones such as estrogen is markedly reduced with a concomitant increase in cardiovascular events, which suggests that estrogen may have a cardioprotective effect.7,8 With regard to the relationship between estrogen and NO, it has been demonstrated that estrogen stimulated constitutive NO synthase activity in cultured endothelial cells.9 Cicinelli et al10 reported that hormone replacement therapy significantly increased the plasma level of the NO metabolites in postmenopausal women. These findings suggest that NO might be involved in the regulation of cardiovascular functions in postmenopausal women, although the precise role of NO in the pathophysiology of hypertension in postmenopausal women is not fully understood.

It has been proposed that cell membrane abnormalities of erythrocytes are an etiologic factor in hypertension, including not only functional abnormalities such as transmembrane cation fluxes11,12 but also structural changes in

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cell membranes. Recently it has been shown that NO may actively participate in the regulation of membrane properties of erythrocytes. In an in vitro study, we demonstrated that exogenously applied NO donor improved membrane fluidity of erythrocytes in essential hypertension, which indicated that NO might have a beneficial effect on rheologic behavior of erythrocytes and microcirculation in hypertension. In the present study, to gain further insight into the possible role of NO in the regulation of membrane functions, we have examined the relationship between plasma level of the NO metabolites (nitrite and nitrate) and membrane fluidity of erythrocytes in hypertensive and normotensive postmenopausal women by means of the EPR method.

Materials and Methods

Subjects

A total of 16 postmenopausal women with essential hypertension were studied and compared with 20 age-matched normotensive postmenopausal women (Table 1). Consent was obtained from all participants after the nature and objective of the study was explained. The hypertensive group consisted of borderline and mild hypertensive patients and they had taken no medication for at least 12 weeks before the study. Participants had not undergone surgically induced menopause, had not had a menstrual period in the preceding year, had not received hormone replacement therapy, and had plasma 17β-estradiol (E2) level of <20 pg/mL. All women in this study had similar lifestyles and dietary habits and were instructed to avoid any changes in dietary habits at least 12 weeks before the study.

EPR Measurements of Membrane Fluidity of Erythrocytes in Postmenopausal Women

Blood samples were obtained by venipuncture after a minimum of 30 min bed rest while fasting. 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). The erythrocyte suspension (erythrocytes 100 μL and Tris-HCl buffer 200 μL: 300 μL total) was incubated for 2 h at 37°C with 100 μL of the solution containing fatty acid spin label agents (5-nitroxide stearate; 5-NS 5×10−5 mol/L). The EPR measurements were then performed using an EPR spectrometer (model Jeol JES-FE2XG, Nihon Denshi, Tokyo, Japan) with a microwave unit (model Jeol ES-SCXA, Nihon Denshi). The microwave power was 5 mW and the modulation frequency 100 KHz with a modulation amplitude of 2.0 gauss. 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 4.0×103−7.9×103 with a response time of 1.0 sec. The fatty acid spin label agents are believed to be anchored at the lipid−aqueous interface of the cell membranes by their carboxyl ends, whereas the nitroxide group moves rapidly through a restricted angle around the point of attachment. Therefore, the EPR spectra of the fatty acid spin label agents are used to detect an alteration in the

Table 1. Clinical characteristics and laboratory findings for hypertensive and normotensive postmenopausal women

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>HT</th>
<th>NT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of subjects</td>
<td>16</td>
<td>20</td>
</tr>
<tr>
<td>Age (y)</td>
<td>64 ± 3</td>
<td>63 ± 3</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>23.4 ± 0.7</td>
<td>23.9 ± 0.9</td>
</tr>
<tr>
<td>Systolic blood pressure (mm Hg)</td>
<td>158.3 ± 3.0*</td>
<td>128.3 ± 2.0</td>
</tr>
<tr>
<td>Diastolic blood pressure (mm Hg)</td>
<td>90.0 ± 2.3*</td>
<td>74.0 ± 1.8</td>
</tr>
<tr>
<td>Heart rate (beats/min)</td>
<td>77.3 ± 3.0*</td>
<td>75.0 ± 3.0</td>
</tr>
<tr>
<td>Erythrocyte counts (10⁴ cells/μL)</td>
<td>437 ± 10</td>
<td>438 ± 11</td>
</tr>
<tr>
<td>Hemoglobin (g/dL)</td>
<td>13.3 ± 0.3</td>
<td>13.7 ± 0.3</td>
</tr>
<tr>
<td>Hematocrit (%)</td>
<td>40.0 ± 0.8</td>
<td>40.9 ± 0.9</td>
</tr>
<tr>
<td>Leukocyte counts (10³ cells/μL)</td>
<td>5.6 ± 0.4</td>
<td>5.6 ± 0.3</td>
</tr>
<tr>
<td>Platelets (10⁴ cells/μL)</td>
<td>23.1 ± 1.3</td>
<td>23.5 ± 1.1</td>
</tr>
<tr>
<td>Total cholesterol (mg/dL)</td>
<td>220 ± 9</td>
<td>225 ± 7</td>
</tr>
<tr>
<td>HDL cholesterol (mg/dL)</td>
<td>55 ± 3</td>
<td>57 ± 4</td>
</tr>
<tr>
<td>LDL cholesterol (mg/dL)</td>
<td>142 ± 8</td>
<td>143 ± 6</td>
</tr>
<tr>
<td>Triglycerides (mg/dL)</td>
<td>120 ± 10</td>
<td>142 ± 14</td>
</tr>
<tr>
<td>Serum sodium (mmol/L)</td>
<td>141.2 ± 0.4</td>
<td>140.9 ± 0.4</td>
</tr>
<tr>
<td>Serum potassium (mmol/L)</td>
<td>4.3 ± 0.2</td>
<td>4.3 ± 0.1</td>
</tr>
<tr>
<td>Serum creatinine (mg/dL)</td>
<td>0.7 ± 0.1</td>
<td>0.7 ± 0.1</td>
</tr>
<tr>
<td>Plasma E₂ concentration (pg/mL)</td>
<td>4.5 ± 1.4</td>
<td>4.0 ± 1.0</td>
</tr>
</tbody>
</table>

HT = postmenopausal women with essential hypertension; NT = normotensive postmenopausal women.

P < .05 between HT and NT.

* Values are means ± SEM.
freedom of motion in biologic membranes and to provide an indication of membrane fluidity.\textsuperscript{20,21} For an index of membrane fluidity, we have evaluated the values of outer and inner hyperfine splitting ($2T_\text{r}$ and $2T'_\text{r}$ in G, respectively) in the EPR spectra and calculated the order parameter ($S$) from $2T_\text{r}$ and $2T'_\text{r}$.\textsuperscript{17,19–21} The greater the value of the order parameter ($S$), the lower the freedom of motion of the spin labels in the biomembrane bilayers indicating lower membrane fluidity.\textsuperscript{17–21}

**NO Metabolites (Nitrite and Nitrate) Analysis**

The plasma level of the NO metabolites (nitrite and nitrate) was measured with an automated NO detector/high performance liquid chromatography system (ENO 10, Eicom Co., Tokyo, Japan), as previously described.\textsuperscript{27} Briefly, nitrite and nitrate in plasma were separated by a reverse-phase separation column, and the nitrate was reduced to nitrite in a reduction column. Nitrite was mixed with Griess reagents (sulfanilamide and naphthalene-ethylene diamine dihydrochloride), and the absorbance at 540 nm was measured by a flow-through spectrometer.

**Measurement of Plasma 17β-Estradiol Concentration**

The plasma 17β-estradiol ($E_2$) concentration was measured with a radioimmunoassay kit (Shionogi Co., Tokyo, Japan).

**Study Drugs**

The spin label agent (5-NS) was purchased from Aldrich (Milwaukee, WI). All other drugs were standard laboratory reagents of analytical grade.

**Statistical Analysis**

Values are expressed as mean ± SEM. The differences between hypertensive and normotensive postmenopausal women were analyzed by the unpaired Student $t$ test. Linear regression analysis was used to identify the correlation between membrane fluidity of erythrocytes (order parameter $S$) and plasma level of the NO metabolites (nitrite and nitrate). A $P$ value < .05 was accepted as the level of significance.

**Results**

**Membrane Fluidity of Erythrocytes and Plasma Level of NO Metabolites (Nitrite and Nitrate) in Hypertensive and Normotensive Postmenopausal Women**

The value of the order parameter ($S$) in the EPR spectra was significantly greater in hypertensive postmenopausal women than in normotensive postmenopausal women ($S$ value: HT $0.726 ± 0.003, n = 16$, NT $0.712 ± 0.002, n = 20$, $P < .05$). The finding indicated that the erythrocyte membrane fluidity was decreased in hypertensive postmenopausal women compared with normotensive postmenopausal women. On the other hand, the plasma level of the NO metabolites (nitrite and nitrate) was significantly lower in hypertensive postmenopausal women than in normotensive postmenopausal women (HT $27.8 ± 2.9 \mu$mol/L, $n = 16$, NT $46.7 ± 5.3 \mu$mol/L, $n = 20$, $P < .05$). The plasma concentration of 17β-estradiol ($E_2$) as well as other routine laboratory findings were not different between hypertensive and normotensive postmenopausal women (Table 1).

**Relationship Between Membrane Fluidity of Erythrocytes and Plasma Level of NO Metabolites (Nitrite and Nitrate) in Hypertensive and Normotensive Postmenopausal Women**

Fig. 1 demonstrated the relationship between membrane fluidity of erythrocytes and plasma level of the NO metabolites (nitrite and nitrate) in postmenopausal women. The order parameter ($S$) in the EPR spectra of erythrocyte membranes was inversely correlated with the plasma level of the NO metabolites in the overall analysis of the hypertensive and normotensive postmenopausal women. The results indicated that the lower membrane fluidity of erythrocytes was associated with the lower plasma NO level in postmenopausal women. When the analysis of the correlation for the hypertensive and normotensive women was performed separately, there was a significant inverse correlation between the order parameter ($S$) of erythrocytes and plasma level of the NO metabolites in the normotensive group ($r = −0.47, n = 20$, $P < .05$) but not in the hypertensive group.

There was no significant correlation between the membrane fluidity of erythrocytes and the plasma 17β-estradiol
Discussion

There has been much evidence showing that NO may actively participate in the pathophysiology of hypertension. In the present study, we determined a role of NO in the regulation of membrane fluidity of erythrocytes in hypertensive and normotensive postmenopausal women by means of the EPR and spin labeling method. The value of the order parameter (S) obtained from the EPR spectra of erythrocyte membranes was significantly greater in hypertensive postmenopausal women than in normotensive postmenopausal women. The results suggest that the membrane fluidity of erythrocytes was lower in hypertensive postmenopausal women than in normotensive postmenopausal women, and confirm our previous reports showing that the cell membranes were stiffer and less fluid in primary hypertension. If the deformability of erythrocytes is highly dependent on the membrane fluidity, the reduction in membrane fluidity could cause a disturbance in blood rheologic behavior and microcirculation, which might contribute to the pathophysiology of hypertension.

The present study also demonstrated that the plasma level of the NO metabolites (nitrite and nitrate) was significantly lower in hypertensive postmenopausal women than in normotensive postmenopausal women. Although the sources of plasma NO are not fully understood, the decreased circulating plasma NO may result from a diffuse endothelial dysfunction throughout the body in hypertensive postmenopausal women. In addition, we showed that the order parameter (S) in the EPR spectra of erythrocyte membranes was inversely correlated with the plasma level of the NO metabolites in postmenopausal women. The results indicate that the lower membrane fluidity of erythrocytes was associated with the lower plasma NO level, and further support the idea that NO might be a determinant factor of the membrane fluidity of erythrocytes in postmenopausal women. When the analysis of the correlation for the hypertensive and normotensive women was performed separately, there was a significant correlation between membrane fluidity of erythrocytes and plasma level of the NO metabolites in the normotensive group but not in the hypertensive group. Although our analysis was restricted to a small population of Japanese women and the findings may not be generalized to other ethnicities, it is possible that NO might help to determine the membrane fluidity of erythrocytes in the normotensive status rather than in the hypertensive status.

The precise mechanisms underlying the effects of NO on the membrane fluidity are still uncertain. Jubelin and Gierman have shown that erythrocytes of rats and humans are positive for NO synthase, which might indicate that erythrocytes possess all the cellular machinery to synthesize their own NO. They proposed that erythrocytes would synthesize and use NO to modulate their own physiology. Chen and Mehta also provided direct evidence showing that human erythrocytes possess endothelium-type NO synthase in the cytosol. We also demonstrated that exogenously applied NO donor (S-nitroso-N-acetylpenicillamine) significantly increased membrane fluidity of erythrocytes in essential hypertension in vitro. It would be possible that the membrane action of NO could be one of the mechanisms responsible for its beneficial effects in improving the rheologic behavior of erythrocyte membranes and the microcirculation in postmenopausal women.

There is a strong link between menopause and an increased incidence of cardiovascular diseases, and recent studies have demonstrated that estrogen may have a cardioprotective effect. It has also been demonstrated that estrogen stimulated NO production both in vitro and in vivo. Higashi et al. reported that forearm blood flow measured by strain gauge plethysmography during reactive hyperemia was significantly lower in hypertensive postmenopausal women than in normotensive postmenopausal women. The finding proposed that endothelial function might be disturbed in hypertensive postmenopausal women, as forearm microcirculation is largely dependent on release of NO from the vascular endothelium. Although the precise role of NO in the regulation of membrane fluidity is still unclear, one hypothesis is that the combination of estrogen deficiency resulting from menopause and hypertension might reduce NO production, which could at least partially explain the decrease in membrane fluidity of erythrocytes in hypertensive postmenopausal women. Further studies should be conducted to assess more thoroughly the relationships between NO and membrane fluidity of erythrocytes and their contribution to the pathophysiology of hypertension in postmenopausal women.

In summary, the results of the present study showed that the lower membrane fluidity of erythrocytes was associated with the lower plasma NO level in postmenopausal women. The results also support the hypothesis that NO may constitute a crucial effect on the physicochemical properties of the cell membranes and the microcirculation in postmenopausal women.

References