Electron Paramagnetic Resonance Investigation on Modulatory Effect of 17β-Estradiol on Membrane Fluidity of Erythrocytes in Postmenopausal Women

Kazushi Tsuda, Yukiko Kinoshita, Keizo Kimura, Ichiro Nishio, Yoshiaki Masuyama

Abstract—Many studies have shown that estrogen may exert cardioprotective effects and reduce the risk of hypertension and coronary events. On the other hand, it has been proposed that cell membrane abnormalities play a role in the pathophysiology of hypertension, although it is not clear whether estrogen would influence membrane function in essential hypertension. The present study was performed to investigate the effects of 17β-estradiol (E2) on membrane fluidity of erythrocytes in normotensive and hypertensive postmenopausal women. We determined the membrane fluidity of erythrocytes by means of an electron paramagnetic resonance and spin-labeling method. In an in vitro study, E2 significantly decreased the order parameter for 5-nitroxide stearate and the peak height ratio for 16-nitroxide stearate obtained from electron paramagnetic resonance spectra of erythrocyte membranes in normotensive postmenopausal women. The finding indicates that E2 might increase the membrane fluidity of erythrocytes. The effect of E2 was significantly potentiated by the NO donor, S-nitroso-N-acetylpenicillamine, and a cGMP analogue, 8-bromo-cGMP. In contrast, the change in the membrane fluidity evoked by E2 was attenuated in the presence of the NO synthase inhibitor, N\textsubscript{G} nitro- L -arginine methyl ester, and asymmetric dimethyl-L-arginine. In hypertensive postmenopausal women, the membrane fluidity of erythrocytes was significantly lower than that in normotensive postmenopausal women. The effect of E2 on membrane fluidity was significantly more pronounced in the erythrocytes of hypertensive postmenopausal women than in the erythrocytes of normotensive postmenopausal women. The results of the present study showed that E2 significantly increased the membrane fluidity and improved the microviscosity of erythrocyte membranes, partially mediated by an NO- and cGMP-dependent pathway. Furthermore, the greater action of E2 in hypertension might be consistent with the hypothesis that E2 could have a beneficial effect in regulating rheological behavior of erythrocytes and could have a crucial role in the improvement of the microcirculation in hypertension.


Key Words: 17β-estradiol ■ nitric oxide ■ membrane fluidity ■ erythrocytes ■ postmenopausal women

There is a strong link between menopause and an increased incidence of cardiovascular diseases, and recent studies have demonstrated that estrogen replacement therapy reduced morbidity or mortality due to coronary heart diseases in postmenopausal women.\textsuperscript{1–4} It has also been shown that estrogen supplementation induced a significant fall in blood pressure in postmenopausal women.\textsuperscript{5} These findings propose the idea that estrogen may exert protective effects against arteriosclerosis and hypertension. However, fundamental mechanisms of cellular and molecular events by estrogen are still unclear.

It has been proposed that cell membrane abnormalities are an etiological factor in hypertension, including functional abnormalities, such as transmembrane cation fluxes.\textsuperscript{6–8} 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.\textsuperscript{9} The membrane fluidity is a physicochemical feature of biomembranes and is an important factor in modulating the cell rheological behavior.\textsuperscript{10} We have shown previously that the membrane fluidity of erythrocytes is significantly lower in spontaneously hypertensive rats and in patients with essential hypertension than in the normotensive controls,\textsuperscript{11–14} and we have proposed that abnormal microviscosity of the cell membranes might contribute to blood pressure elevation. However, it is not clear whether estrogen influences the membrane fluidity of erythrocytes. In the present study, to assess the modulatory action of estrogen on the membrane function, we investigated the effects of 17β-estradiol (E2) on the membrane fluidity of erythrocytes in normotensive and hypertensive postmenopausal women by means of the EPR and spin-labeling method.

Methods

Study I

Subjects
To investigate the effects of E2 on the membrane fluidity in vitro, erythrocytes were obtained from postmenopausal women who were...
normotensive volunteers (n=78, aged 61±1 [mean±SEM] years, blood pressure 130.0±2.4/79.1±1.5 mm Hg, heart rate 76.8±1.4 bpm, body mass index 22.8±0.3 kg/m², and plasma E₂ concentration 4.5±0.7 pg/mL). Written informed consent was obtained from all volunteers.

**Effects of E₂ Alone on Membrane Fluidity of Erythrocytes in Normotensive Postmenopausal Women In Vitro**

Blood samples were obtained in patients by venipuncture after a minimum of 30 minutes of bed rest while fasting. After plasma and Buffy coat were carefully removed by centrifugation at 155g for 10 minutes at 4°C, washed erythrocytes were resuspended in the isotonic buffer (140 mmol/L NaCl and 20 mmol/L Tris-HCl, pH 7.4) at a hematocrit of 50%. The erythrocyte suspension (100 µL erythrocytes and 100 µL Tris-HCl buffer, 200 µL total) was incubated for 2 hours at 37°C in the NaCl-Tris buffer (100 µmol/L) alone or 17α-estradiol (1×10⁻⁹ to 1×10⁻⁶ mol/L) alone, because the preliminary examination demonstrated that the maximal effect of E₂ on the membrane fluidity of erythrocytes was obtained after a 2-hour incubation at 37°C. After incubation with E₂, 100 µL of the solution containing fatty acid spin-label agents (5-nitroxide stearate [5-NS] and 16-nitroxide stearate [16-NS], 5×10⁻⁵ mol/L) was added to the erythrocyte suspension (300 µL). The mixed solution was then incubated for 2 hours at 37°C with gentle shaking, and the EPR measurements were performed.

**Effects of E₂ in Combination With SNAP and 8-Bromo-cGMP on Membrane Fluidity of Erythrocytes in Normotensive Postmenopausal Women In Vitro**

To examine the effects of E₂ in combination with an NO donor and cGMP, erythrocytes (100 µL) were pretreated with the same volume of Tris-HCl solution containing 5-nitroso-N-acetylpenicillamine (SNAP) or a cGMP analogue (8-bromo-cGMP) before the application of E₂. After a 2-hour incubation with 100 µL E₂ (1×10⁻⁷ to 1×10⁻⁶ mol/L) at 37°C, 100 µL of the solution containing fatty acid spin-label agents (5-NS and 16-NS, 5×10⁻⁵ mol/L) was added to the erythrocyte suspension (300 µL). The mixed solution was then incubated for 2 hours at 37°C with gentle shaking, and the EPR measurements were performed.

**Effects of E₂ in Combination With L-NAME and ADMA on Membrane Fluidity of Erythrocytes in Normotensive Postmenopausal Women**

To examine the effects of E₂ in combination with an NO donor and ADMA, erythrocytes (100 µL) were pretreated with the same volume of Tris-HCl solution containing L-NAME (1×10⁻⁵ mol/L) or ADMA (1×10⁻⁶ mol/L) before the application of E₂ (1×10⁻⁷ to 1×10⁻⁶ mol/L) at 37°C, 100 µL of the solution containing fatty acid spin-label agents (5-NS and 16-NS, 5×10⁻⁵ mol/L) was added to the erythrocyte suspension (300 µL). The mixed solution was then incubated for 2 hours at 37°C with gentle shaking, and the EPR measurements were performed.

**EPR Measurements of Erythrocytes**

The EPR measurements were performed by using an EPR spectrometer (model Joel ES-FE2XG, Nihon Denshi) with a microwave unit (model Joel ES-SCXA, Nihon Denshi). The microwave power was 5 mW, and the modulation frequency was 100 KHz, with a modulation amplitude of 2.0 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 minutes, and receiver gain was 4.0×10³ to 7.9×10³, with a response time of 1.0 second.

The fatty acid spin-label agents (5-NS and 16-NS) 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 freedom of motion in biological membranes and to provide an indication of membrane fluidity. In addition, 5-NS could serve as an example of the properties of superficial membrane layers, whereas 16-NS could be an indicator referring to more hydrophobic core of the lipid membranes. For indexes of membrane fluidity, we have evaluated the values of outer and inner hyperfine splitting (2T' and 2T-1, in gauss, respectively) in the EPR spectra for 5-NS and calculated the order parameter from 2T' and 2T-1. In the EPR spectra for 16-NS, we used the peak height ratio (ho/h-1) for an index of the membrane fluidity. The greater the values of the order parameter and ho/h-1, the lesser is the freedom of motion of the spin labels in the biomembrane bilayers, indicating lower membrane fluidity.

**Study II**

**Subjects and Protocol**

Twenty-eight postmenopausal women with mild essential hypertension were studied and compared with 33 age-matched normotensive postmenopausal women. The characteristics of the hypertensive patients and normotensive subjects are given in the Table. Written informed consent was obtained from all participants after they were informed about the nature and objective of the study. All hypertensive patients had no cardiovascular complications and had no medication at least 4 weeks before the EPR study. In addition, they had no other diseases, such as hematological or hepatic disorders. The effect of E₂ (1×10⁻⁷ and 1×10⁻⁶ mol/L) on membrane fluidity of erythrocytes in vitro was compared between hypertensive and normotensive subjects by means of EPR and the spin-labeling method.

**Measurement of Plasma E₂ Concentration**

Plasma E₂ concentration was measured with a radioimmunosay kit (Shionogi Co, Ltd).

**Drugs**

E₂ was obtained from Biomedical Technologies Inc, and its stereoisomer, 17α-estradiol, was obtained from Sigma Chemical Co. The spin label agents, 5-NS and 16-NS, were purchased from Aldrich Co, Ltd. SNAP, 8-bromo-cGMP, L-NAME, and ADMA were obtained from Funakoshi Co, Ltd. All other drugs were standard laboratory reagents of analytical grade.

**Statistical Analysis**

Values are expressed as mean±SEM. The differences between the means of the drug treatment and their corresponding controls were

| Clinical Characteristics and Laboratory Findings for NT and HT Groups |
|------------------|------------------|
|                  | HT               | NT               |
| n                | 28               | 33               |
| Age, y           | 62±2             | 61±2             |
| BMI, kg/m²       | 23.2±0.5         | 22.4±0.3         |
| Systolic blood pressure, mm Hg | 154.9±2.8* | 120.4±1.6 |
| Diastolic blood pressure, mm Hg | 90.7±1.4* | 69.6±1.2 |
| Heart rate, bpm  | 79.0±2.0         | 75.0±2.0         |
| Erythrocyte counts, 10⁴ cells/µL | 431±7 | 430±6 |
| Hemoglobin, g/dL | 13.1±0.2         | 13.4±0.2         |
| Hematocrit, %    | 39.1±0.6         | 40.4±0.5         |
| Leukocyte counts, 10³ cells/µL | 6.0±0.3 | 6.2±0.2 |
| Platelets, 10⁴ cells/µL | 23.2±0.8 | 24.6±0.7 |
| Total cholesterol, mg/dL | 226.2±6.0 | 226.2±4.0 |
| Triglycerides, mg/dL | 117.4±12.0 | 137.6±10.4 |
| Serum creatinine, mg/dL | 0.7±0.1 | 0.7±0.1 |
| Plasma E₂ concentration, pg/mL | 4.7±1.0 | 4.5±1.2 |

*P<0.05 between HT and NT.
tested with a 1-way ANOVA. To compare the means of the different study groups, the Wilcoxon signed rank sum test was used. The differences between hypertensive and normotensive postmenopausal women were analyzed with a 2-way ANOVA, followed by the Mann-Whitney U test. A value of $P < 0.05$ was accepted as the level of significance.

**Results**

**Effects of E2 Alone on Membrane Fluidity of Erythrocytes in Normotensive Postmenopausal Women In Vitro**

E2 ($1 \times 10^{-9}$ to $1 \times 10^{-6}$ mol/L) decreased the order parameter for 5-NS and peak height ratio (ho/h-1) for 16-NS obtained from erythrocyte membranes in normotensive volunteers in a dose-dependent manner (order parameter was as follows: control, 0.716±0.001 [n=52]; 1×10⁻⁹ mol/L E₂, 0.696±0.001 [n=52], $P<0.01$; 1×10⁻⁸ mol/L E₂, 0.688±0.002 [n=52], $P<0.01$; 1×10⁻⁷ mol/L E₂, 0.683±0.001 [n=52], $P<0.01$; and 1×10⁻⁶ mol/L E₂, 0.679±0.002 [n=52], $P<0.01$; ho/h-1 was as follows: control, 5.10±0.02 [n=52]; 1×10⁻⁹ mol/L E₂, 4.93±0.02 [n=52], $P<0.01$; 1×10⁻⁸ mol/L E₂, 4.85±0.02 [n=52], $P<0.01$; 1×10⁻⁷ mol/L E₂, 4.78±0.02 [n=52], $P<0.01$; and 1×10⁻⁶ mol/L E₂, 4.74±0.02 [n=52], $P<0.01$). This finding shows that E₂ increased the membrane fluidity of erythrocytes. On the other hand, 17α-estradiol, the stereoisomer of E₂, showed no significant effects of membrane fluidity of erythrocytes (order parameter was as follows: control, 0.711±0.004 [n=5]; 1×10⁻⁷ mol/L 17α-estradiol, 0.711±0.006 [n=5]; 1×10⁻⁶ mol/L 17α-estradiol, 0.715±0.008 [n=5]; and 1×10⁻⁵ mol/L 17α-estradiol, 0.715±0.009 [n=5]). ho/h-1 was as follows: control, 5.24±0.08 [n=5]; 1×10⁻⁷ mol/L 17α-estradiol, 5.30±0.12 [n=5]; 1×10⁻⁶ mol/L 17α-estradiol, 5.30±0.11 [n=5]; and 1×10⁻⁵ mol/L 17α-estradiol, 5.29±0.10 [n=5]).

**Effects of E₂ in Combination With SNAP or 8-Bromo-cGMP on Membrane Fluidity of Erythrocytes**

A preliminary study showed that SNAP alone reduced the values of the order parameter and ho/h-1 of erythrocyte membranes (order parameter was as follows: control, 0.716±0.004 [n=6]; 5×10⁻⁶ mol/L SNAP, 0.712±0.007 [n=6], $P<0.05$; ho/h-1 was as follows: control, 5.15±0.03 [n=6]; 5×10⁻⁶ mol/L SNAP, 5.11±0.06 [n=6], and 5×10⁻⁷ mol/L SNAP, 4.76±0.09 [n=6], $P<0.05$). In the present experiment, it was clearly demonstrated that the effect of E₂ on the fluidity was significantly potentiated by a low concentration of SNAP (5×10⁻⁶ mol/L), which showed no effects of its own (Figure 1).

Similarly, the cGMP analogue, 8-bromo-cGMP, reduced the values of the order parameter and ho/h-1 of erythrocyte membranes (order parameter was as follows: control, 0.704±0.004 [n=6]; 1×10⁻⁶ mol/L 8-bromo-cGMP, 0.703±0.002 [n=6], and 1×10⁻⁵ mol/L 8-bromo-cGMP, 0.686±0.004 [n=6], $P<0.05$; ho/h-1 was as follows: control, 5.31±0.05 [n=6]; 1×10⁻⁶ mol/L 8-bromo-cGMP, 5.30±0.05 [n=6], and 1×10⁻⁵ mol/L 8-bromo-cGMP, 5.08±0.06 [n=6], $P<0.05$). The effect of E₂ on the fluidity was significantly enhanced in the presence of a low concentration (1×10⁻⁶ mol/L) of 8-bromo-cGMP (Figure 2), although this concentration of 8-bromo-cGMP alone showed no significant effects on the membrane fluidity of its own.

**Effects of E₂ in Combination With L-NAME and ADMA on Membrane Fluidity of Erythrocytes**

Figure 3 shows the effects of E₂ on the membrane fluidity of erythrocytes in the presence of L-NAME (1×10⁻⁵ mol/L).
The effect of E2 was significantly attenuated in the presence of L-NAME. Similarly, ADMA (1 × 10^{-4} mol/L) significantly counteracted the E2-induced changes in membrane fluidity of erythrocytes (Figure 4).

Membrane Fluidity of Erythrocytes in Postmenopausal Women With Essential Hypertension and Normotensive Postmenopausal Women

The values of the order parameter and ho/h-1 of the EPR spectra were significantly greater in postmenopausal women with essential hypertension than in age-matched normotensive postmenopausal women (order parameter was as follows: hypertensive group, 0.722±0.002 [n=28]; normotensive group, 0.712±0.002 [n=33], P<0.01; ho/h-1 was as follows: hypertensive group, 5.25±0.03 [n=28]; normotensive group, 5.10±0.02 [n=33], P<0.01). The finding indicated that the erythrocyte membrane fluidity was decreased in postmenopausal women with essential hypertension compared with normotensive postmenopausal women.
Effects of E2 on Membrane Fluidity of Erythrocytes in Postmenopausal Women With Essential Hypertension and Normotensive Postmenopausal Women

The preliminary study showed that the effect of E2 on membrane fluidity of erythrocytes was also reversed in the presence of L-NAME in hypertensive postmenopausal women (order parameter was as follows for 10 postmenopausal women (percent change in order parameter (increased the membrane fluidity) to a greater extent was as follows: for 10 hypertensive postmenopausal women than in normotensive postmenopausal women (percent change in order parameter was as follows: control, 5.38 ± 0.05 [n = 7]; 10^−7 mol/L E2, 6.08 ± 0.03 [n = 7], P < 0.05 versus control; 10^−6 mol/L E2, 6.85 ± 0.04 [n = 7], P < 0.05 versus control; 10^−5 mol/L L-NAME alone, 5.72 ± 0.03 [n = 7], P < 0.05 versus control; and 10^−6 mol/L E2 plus 10^−5 mol/L L-NAME, 5.72 ± 0.04 [n = 7], P < 0.05 versus control). For 5-NS and the peak height ratio (ho/h−1) for 16-NS showed no significant effects on membrane fluidity. These findings indicated that E2 significantly increased the membrane fluidity of erythrocytes in postmenopausal women.

Because membrane fluidity is inversely correlated with membrane microviscosity, it would be possible that the membrane action of E2 could be one of the mechanisms responsible for its beneficial effects in improving the rheological behavior of erythrocyte membranes. In the present study, we used a concentration range of 10^−9 to 10^−6 mol/L for E2. The concentrations might be higher than those expected by the endogenous E2 content in human plasma. However, in an in vitro preparation, higher dosages were necessary because the compound could be gradually inactivated by degradation.

It is well recognized that signal transduction induced by estrogen is mediated through intranucleus estrogen receptors (genomic receptors). Recently, it has also been shown that nongenomic estrogen receptors are present on the membranes. It was reported that when erythrocytes were incubated with estrogen in vitro, two thirds was bound to the membrane, whereas one third was in the soluble fraction.
Puca and Sica\textsuperscript{21} provided evidence for the existence of specific and high-affinity binding components to estrogen in the cytoskeletal matrix of the erythrocyte membranes. However, it is still uncertain whether erythrocytes might bear the specific receptors for estrogen, and the nonspecific action of estrogen cannot be fully excluded.

In the present study, it has also been clearly shown that the effect of E\textsubscript{2} is significantly potentiated by a low concentration of SNAP, an NO donor, and a cGMP-analogue, 8-bromo-cGMP, which have no effects by themselves. These synergistic effects suggest that the action of E\textsubscript{2} might be, at least in part, mediated by the NO- and cGMP-related pathway. The hypothesis was confirmed by the finding that the effects of E\textsubscript{2} were blocked by L-NAME and ADMA, the NO synthesis inhibitors. NO is a potent stimulator of guanylate cyclase activity and is produced by different isoforms of NO synthase.\textsuperscript{22} Jubelin and Gierman\textsuperscript{23} have shown that erythrocytes of rats and humans are positive for NO synthase, which indicates 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. We also reported that NO might have a crucial role in the regulation of the membrane fluidity of erythrocytes.\textsuperscript{24} In other tissues, it has been demonstrated that the effects of estrogen might, at least in part, be mediated by the production of NO.\textsuperscript{25–28} These previous findings coupled with our present results suggest that NO might play a role in estrogen-induced alterations in membrane properties, although further studies should be conducted to assess more thoroughly the relationship between NO and estrogen effects on the membrane function.

The values of the order parameter and h\textsubscript{o}/h\textsubscript{1} obtained from the erythrocyte EPR spectra were significantly greater in postmenopausal women with essential hypertension 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.\textsuperscript{11–15} If the deformability of erythrocytes is highly dependent on the membrane fluidity,\textsuperscript{10,29} the reduction in membrane fluidity could cause a disturbance in the blood rheological behavior and in the microcirculation, which might contribute to the pathophysiology of hypertension. The present study also demonstrated that E\textsubscript{2} increased the membrane fluidity of erythrocytes to a greater extent in hypertensive postmenopausal women than in normotensive postmenopausal women. The finding might be consistent with our previous report showing that the effect of the NO donor, SNAP, on the erythrocyte membrane fluidity was more pronounced in patients with essential hypertension than in normotensive subjects.\textsuperscript{24} Although the precise role of estrogen in the regulation of membrane fluidity in hypertension is still unclear, one hypothesis is that estrogen may improve membrane fluidity and contribute to the defense against a further increase in microviscosity in hypertension.

In summary, the results of the present study showed that E\textsubscript{2} increased membrane fluidity of erythrocytes in postmenopausal women. The effects were mediated, at least to some extent, by the NO- and cGMP-dependent pathway. Our data also suggest that estrogen may have a crucial modulatory action on erythrocyte membrane fluidity that may also be of considerable biological and clinical significance in determining rheological properties of the membranes. Furthermore, the greater action of E\textsubscript{2} in hypertension might be consistent with the hypothesis that estrogen could have a beneficial effect on erythrocyte membrane function and the microcirculation in hypertensive postmenopausal women.

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