

Hyperhomocysteinemia and Increased Oxidative Stress Levels Are Associated with Impaired Membrane Fluidity of Red Blood Cells in Hypertensive and Normotensive Men: An Electron Spin Resonance Investigation

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ABSTRACT

Hyperhomocysteinemia and oxidative stress may be strongly linked to hypertension, atherosclerosis and other cardiovascular diseases. The present study was performed to investigate possible relationships among plasma total homocysteine, plasma 8-iso-prostaglandin F_{2α} (8-isoPG F_{2α}: an index of oxidative stress), and membrane fluidity (a reciprocal value of membrane microviscosity) in hypertension. We measured the membrane fluidity of red blood cells (RBCs) in hypertensive and normotensive men using an electron spin resonance (ESR) and spin-labeling method. Membrane fluidity of RBCs was significantly decreased in hypertensive men compared with normotensive men. Plasma total homocysteine levels were significantly higher in hypertensive men than in normotensive men, and correlated with plasma 8-isoPG F_{2α}. In contrast, plasma nitric oxide (NO)-metabolites (an index of endothelial function) were lower in hypertensive men than in normotensive men. The reduced membrane fluidity of RBCs was associated with increased total homocysteine and plasma 8-isoPG F_{2α} levels and decreased plasma NO-metabolite levels. Multivariate regression analysis showed that, after adjusting for general risk factors, plasma total homocysteine and 8-isoPG F_{2α} were significant determinants of membrane fluidity of RBCs, respectively. These results suggest that hyperhomocysteinemia and oxidative stress with endothelial dysfunction might have a close correlation with impaired rheologic behavior of RBCs and circulatory disorders in hypertensive men.

Keywords: Homocysteine; Oxidative Stress; 8-Iso-Prostaglandin F_{2α}; Nitric Oxide; Membrane Fluidity; Red Blood Cell; Electron Spin Resonance; Hypertension

1. Introduction

Evidence indicates that many cardiometabolic factors, including hyperhomocysteinemia, might have a pivotal role in the progression of hypertension, atherosclerosis and other cardiovascular diseases [1-3]. Wald *et al.* [2] demonstrated the strong link between homocysteine and cardiovascular diseases, and proposed that lowering plasma homocysteine concentration would reduce the risk of ischemic heart disease, deep vein thrombosis, and stroke. It has also been shown that endothelium-dependent vasodilation was impaired in humans with elevated plasma homocysteine [4], suggesting that hyperhomocysteinemia could reduce the nitric oxide (NO)-availability and actively participate in the pathogenesis of vascular dysfunction.

On the other hand, it is well recognized that oxidative stress might be associated with increased risk of vascular

damage, cardiovascular diseases and the metabolic syndrome [5-9]. Recently, it has been shown that plasma 8-iso-prostaglandin F_{2α} (8-iso-PG F_{2α}) may be a reliable index of oxidative stress in humans [10-14]. It was demonstrated that plasma concentration of 8-iso-PG F_{2α} was significantly increased in subjects with essential hypertension compared with normotensive subjects [10,11,14]. It was also shown that plasma 8-iso-PG F_{2α} levels were elevated in patients with coronary artery disease, especially in those with hypertension [12,13].

Abnormalities in physical properties of the cell membranes may underlie the defects that are strongly linked to hypertension [15,16]. An electron spin resonance (ESR) and spin-labeling method has been developed to evaluate the membrane fluidity (a reciprocal value of membrane microviscosity) and perturbations of the membrane function by external agents [15,16]. Using the ESR method,

we have been performing a series of experiments regarding the membrane fluidity in subjects with essential hypertension [14,17-23]. The results suggest that the membrane fluidity of red blood cells (RBCs) was significantly lower in hypertensive subjects than in normotensive subjects, indicating that the cell membranes were stiffer and less fluid in essential hypertension [14,17-23]. Because the deformability of RBCs may be highly dependent on the membrane fluidity, the reduction in membrane fluidity of RBCs could cause a disturbance in the blood rheologic behavior and in the microcirculation, which might contribute to the pathophysiology of hypertension and other circulatory disorders [15,16]. In the studies presented previously, we showed that hyperhomocysteinemia and oxidative stress might be associated with reduced membrane fluidity of RBCs in hypertensive subjects, respectively [14,17,22,23], although interactions between hyperhomocysteinemia and oxidative stress and their role in the regulation of membrane fluidity of RBCs remain unclear. In the present study, therefore, we investigated possible relationships among plasma total homocysteine levels, oxidative stress and membrane fluidity of RBCs in hypertensive and normotensive men using the ESR method.

2. Subjects and Methods

2.1. Subjects

A total of 32 men with untreated essential hypertension (systolic blood pressure more than 140 mmHg and/or diastolic blood pressure more than 90 mmHg) were studied and compared with 21 age-matched normotensive men. The characteristics and laboratory findings in both groups were shown in **Table 1**. Subjects who had a history of other diseases such as hematologic or hepatic disorders were excluded. All men were non-smokers. They had similar life styles and dietary habits, and were instructed to avoid any changes in dietary habits before the study. The study was approved by a local research committee of Kansai University of Health Sciences. Informed consent was obtained from all participants after they were informed about the nature and objective of the study.

2.2. Electron Spin Resonance Measurements of Red Blood Cells

Blood sampling was performed by venipuncture after a 30 minutes of bed rest while fasting. The procedures of RBC preparation and ESR measurements were shown previously [17-23]. We used heparin as the anti-coagulant (10 U heparin/10 ml blood). The plasma and buffy coat were carefully removed by centrifugation at 155 g for 10 min at 4°C. Then, the sedimented RBCs were re-

suspended in the isotonic buffer (140 mmol/L NaCl 20 mmol/L Tris-HCL, pH 7.4). The RBC solution was centrifuged at 155 g for 10 min at 4°C. This procedure was repeated three times and the washed RBCs were suspended in the isotonic buffer (140 mmol/L NaCl and 20 mmol/L Tris-HCl buffer, pH 7.4) at a hematocrit of 50%. The RBC suspension (300 µl) was incubated with the solution (100 µl) containing fatty acid spin label agent (5-nitroxide stearate 5×10^{-5} mol/L, Aldrich Co. Ltd., Milwaukee, Wisconsin, USA) for 2 hours with gentle shaking at 37°C [17-19]. After incubation, the ESR measurements were immediately performed using an ESR spectrometer (model Jeol JES-FE2XG, Nihon Denshi, Tokyo, Japan) with a microwave unit (model Jeol ES-SCXA, Nihon Denshi) [17-23]. The microwave power was 5mW and the modulation frequency was 100 KHz with a modulation amplitude of 0.2 m tesla (T). The temperature of the measurement was controlled at 30°C. For indices of membrane fluidity, we evaluated the values of outer and inner hyperfine splitting ($2T'_{\parallel}$ and $2T'_{\perp}$ in tesla (T), respectively) in each ESR spectrum (**Figure 1**) and calculated the order parameter (S) [20]. The greater the value of the order parameter (S) was, the lower the membrane fluidity of RBCs was [17-23].

Table 1. Clinical characteristics and laboratory findings of hypertensive (HT) and normotensive (NT) men.

	NT	HT
Number of subjects	21	32
Age (y.o.)	64 ± 3	62 ± 2
Body mass index (kg/m ²)	24.1 ± 0.6	24.1 ± 0.5
Systolic blood pressure (mmHg)	125 ± 2	146 ± 1*
Diastolic blood pressure (mmHg)	70 ± 2	86 ± 1*
Heart rate (beats/min)	76 ± 1	73 ± 1
Erythrocyte counts (10 ⁴ cells/µL)	452 ± 10	480 ± 7
Hemoglobin (g/dL)	14.1 ± 0.3	14.2 ± 0.2
Hematocrit (%)	43.1 ± 1.0	43.0 ± 1.0
Leucocyte counts (10 ³ cells/µL)	5.5 ± 0.2	5.3 ± 0.2
Platelets (10 ⁴ cells/µL)	20 ± 1	22 ± 1
Total cholesterol (mg/dL)	200 ± 6	213 ± 6
High density lipoprotein cholesterol (mg/dL)	50 ± 3	53 ± 3
Low density lipoprotein cholesterol (mg/dL)	123 ± 6	126 ± 5
Triglycerides (mg/dL)	137 ± 17	160 ± 18
Serum sodium (mmol/L)	140.5 ± 0.4	140.0 ± 0.3
Serum potassium (mmol/L)	4.0 ± 0.1	4.0 ± 0.1
Serum creatinine (mg/dL)	0.8 ± 0.1	0.9 ± 0.1
Fasting plasma glucose (mg/dL)	113 ± 6	124 ± 8

Values are means ± SEM. *P < 0.01 between HT and NT.

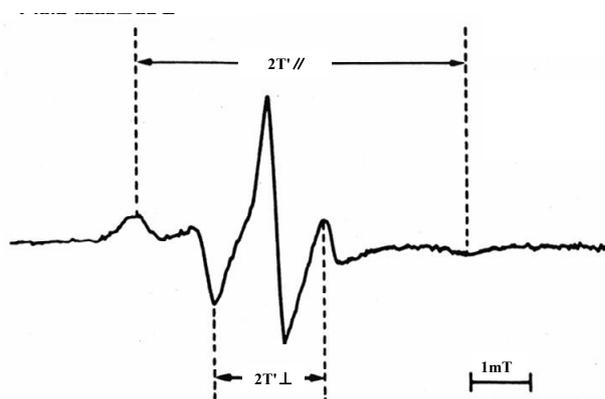


Figure 1. Representative electron spin resonance (ESR) spectrum of red blood cells (RBCs) for the fatty acid spin-label agent (5-nitroxide stearate). $2T'_{//}$: outer hyperfine splitting, $2T'_{\perp}$: inner hyperfine splitting, T: tesla We calculated the order parameter (S) from $2T'_{//}$ and $2T'_{\perp}$ values according to the formula (20). $S = (a_n/a'_n) (T'_{//} - T'_{\perp}) / (T_{zz} - T_{xx})$, a_n/a'_n : isotropic coupling constant, T_{zz} and T_{xx} : hyperfine constants. The greater the value of the order parameter (S) was, the lower the membrane fluidity of RBCs was (17-23).

2.3. Analysis of Plasma 8-Iso-Prostaglandin F_{2α} and Plasma Nitric Oxide-Metabolites (Nitrite and Nitrate)

The levels of plasma 8-iso-PG F_{2α} (an index of oxidative stress) were determined by using an enzyme immunoassay (Cayman Chemicals Co., Ann Arbor, Michigan, USA) [11-13].

The plasma levels of NO-metabolites (nitrite and nitrate) (an index of endothelial function) were measured with an automated NO detector/high-performance liquid chromatography system (ENO 10, Eicom Co., Tokyo, Japan), as previously described [21]. 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.

2.4. Statistical Analysis

Values are expressed as mean \pm SEM. The differences between hypertensive and normotensive men were analyzed using an unpaired Student's t-test. Linear regression analysis was performed to assess the relationships among membrane fluidity (order parameter; S) of RBCs, plasma total homocysteine, plasma 8-iso-PG F_{2α}, and NO-metabolite levels. Multivariate regression analysis with membrane fluidity (order parameter; S) of RBCs as a dependent variable, and general risk factors (age, body

mass index: BMI, plasma total cholesterol, fasting plasma glucose, and systolic blood pressure) and plasma total homocysteine, or plasma 8-iso-PG F_{2α} as independent variables was also performed. A P-value less than 0.05 was accepted as the level of significance.

3. Results

3.1. Membrane Fluidity of Red Blood Cells in Hypertensive and Normotensive Men

The order parameter (S) for the spin-label agents (5-nitroxide stearate) of RBC membranes in the ESR spectra was significantly higher in hypertensive (HT) men than in normotensive (NT) men (HT: 0.728 ± 0.002 , $n = 32$, NT: 0.718 ± 0.001 , $n = 21$, $P < 0.001$). The finding indicated that membrane fluidity was decreased in hypertensive men compared with normotensive men.

3.2. Plasma Total Homocysteine, Plasma 8-Iso-Prostaglandin F_{2α}, and Plasma Nitric Oxide-Metabolite Levels in Hypertensive and Normotensive Men

The plasma total homocysteine levels were significantly elevated in hypertensive men compared with normotensive men (HT: $12.7 \pm 0.8 \mu\text{mol/L}$, $n = 32$, NT: $10.2 \pm 0.4 \mu\text{mol/L}$, $n = 21$, $P < 0.05$). The plasma 8-iso-PG F_{2α} levels were also higher in hypertensive men than in normotensive men (HT: $2.99 \pm 0.26 \text{ nmol/L}$, $n = 32$, NT: $1.96 \pm 0.21 \text{ nmol/L}$, $n = 21$, $P < 0.01$). In contrast, the plasma NO-metabolites were lower in hypertensive men than in normotensive men (HT: $34.5 \pm 2.2 \mu\text{mol/L}$, $n = 32$, NT: $55.4 \pm 4.4 \mu\text{mol/L}$, $n = 21$, $P < 0.001$).

In the overall analysis of hypertensive and normotensive men, plasma total homocysteine levels were correlated with plasma 8-iso-PG F_{2α} ($r = 0.411$, $n = 53$, $P < 0.01$) (Figure 2). In addition, plasma NO-metabolites levels were inversely correlated with and plasma total homocysteine ($r = -0.315$, $n = 53$, $P < 0.05$) (Figure 3), and plasma 8-iso-PG F_{2α} ($r = -0.300$, $n = 53$, $P < 0.05$), respectively.

3.3. Relationship between Membrane Fluidity of Red Blood Cells and Plasma Total Homocysteine, Plasma 8-Iso-Prostaglandin F_{2α}, or Nitric Oxide-Metabolite Levels in Hypertensive and Normotensive Men

The order parameter (S) of RBC membranes in the ESR spectra was correlated with plasma total homocysteine ($r = 0.286$, $n = 53$, $P < 0.05$) (Figure 4) and plasma 8-iso-PG F_{2α} ($r = 0.317$, $n = 53$, $P < 0.05$), and inversely correlated with plasma NO-metabolites ($r = -0.342$, $n =$

53, $P < 0.05$), suggesting that reduced membrane fluidity of RBCs might be associated with hyperhomocysteinemia, increased oxidative stress and endothelial dysfunction.

Multivariate regression analysis also showed that, after adjusting for general risk factors, plasma total homocysteine still remained as a significant determinant of membrane fluidity of RBCs (**Table 2**: Model 1). It was also demonstrated that plasma 8-iso-PG F2 α was an independent predictor of membrane fluidity of RBCs after adjusting for general risk factors (**Table 3**: Model 2).

4. Discussion

There is evidence showing that both hyperhomocysteinemia and oxidative stress might actively participate in the pathophysiology of hypertension, atherosclerosis, and other cardiovascular disease conditions [1-14], although associations of hyperhomocysteinemia and oxidative stress and their role in the regulation of membrane fluidity in hypertension remain to be solved. The present study was performed to evaluate the possible relationships among plasma total homocysteine, plasma 8-iso-PG F2 α and membrane fluidity of RBCs in hypertensive

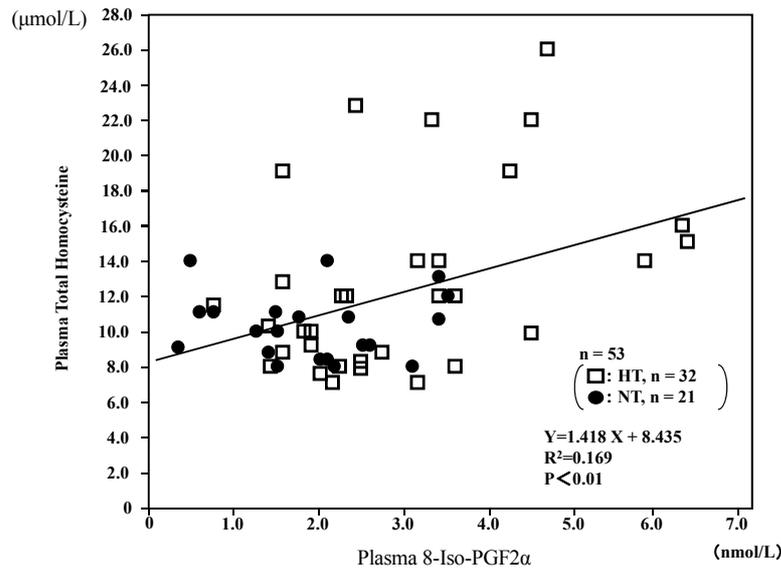


Figure 2. Correlation between plasma 8-iso-prostaglandin F2 α (8-iso-PG F2 α) and plasma total homocysteine levels in hypertensive (HT) and normotensive (NT) men.

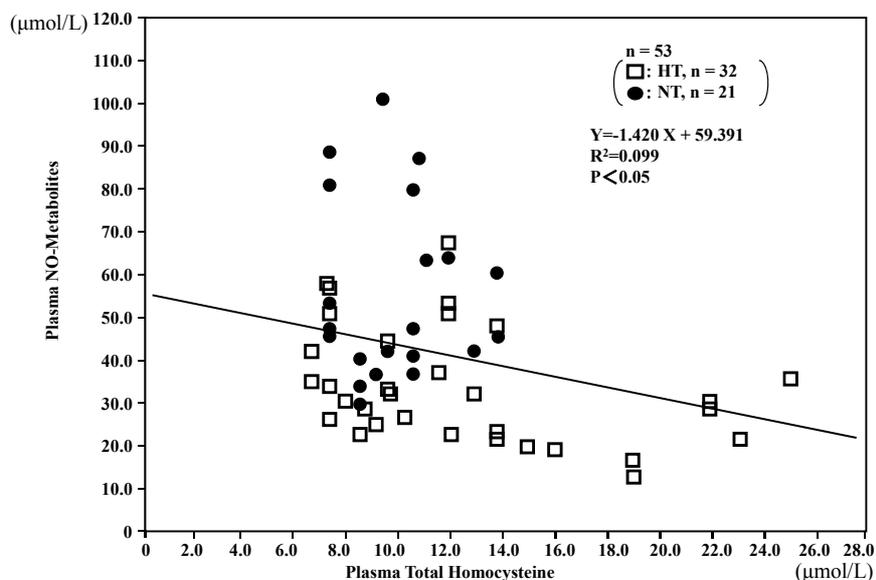


Figure 3. Inverse correlation between plasma total homocysteine levels and plasma nitric oxide (NO)-metabolite levels in hypertensive (HT) and normotensive (NT) men.

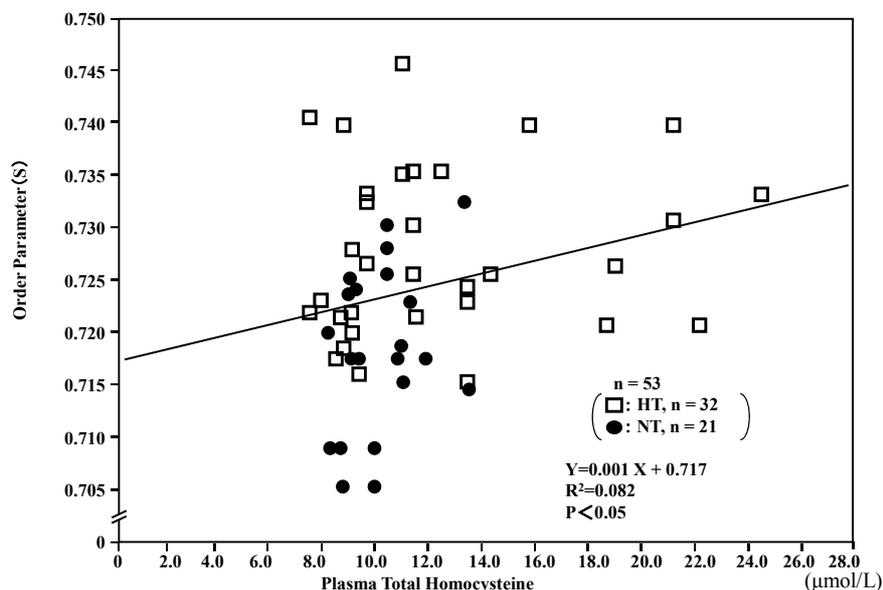


Figure 4. Correlation between plasma total homocysteine levels and membrane fluidity (order parameter; S) of red blood cells (RBCs) in hypertensive (HT) and normotensive (NT) men.

Table 2. Model 1: Multivariate regression analysis of plasma total homocysteine for predicting order parameter (S) of RBCs.

	SRC	t-value	P-value
Age (y.o.)	0.07	0.416	0.6794
Body mass index (kg/m ²)	0.051	0.32	0.7506
Total cholesterol (mg/dL)	-0.137	-0.829	0.4114
Fasting plasma glucose (mg/dL)	0.243	1.71	0.0940
Systolic blood pressure (mm Hg)	0.33	2.423	0.0194
Plasma total homocysteine (µmol/L)	0.35	2.541	0.0145

$R^2 = 0.254$, $n = 53$, $F = 2.610$, $P = 0.0292$; SRC: standard regression coefficient

Table 3. Model 2: Multivariate regression analysis of plasma 8-iso-PGF2 α for predicting order parameter (S) of RBCs.

	SRC	t-value	P-value
Age (y.o.)	6.295×10^{-5}	3.623×10^{-4}	0.9997
Body mass index (kg/m ²)	-0.088	-0.532	0.5970
Total cholesterol (mg/dL)	-0.218	-1.247	0.2186
Fasting plasma glucose (mg/dL)	0.227	1.591	0.1184
Systolic blood pressure (mm Hg)	0.249	1.717	0.0927
Plasma 8-iso-PGF2 α (nmol/L)	0.363	2.335	0.0240

$R^2 = 0.239$, $n = 53$, $F = 2.413$, $P = 0.0413$; RC: standard regression coefficient

and normotensive men using the ESR method. The results showed that the order parameter (S) of RBC membranes in the ESR spectra was significantly higher in hypertensive men than in normotensive men, indicating that membrane fluidity of RBCs was decreased in hypertension. The finding might be consistent with our previous findings showing that the cell membranes were stiffer and less fluid in hypertension [15,17-23].

In the present study, we showed that plasma total homocysteine and plasma 8-iso-PG F2 α (an index of oxidative stress) levels were significantly higher in hypertensive men than in normotensive men. In the overall analysis of hypertensive and normotensive men, plasma total homocysteine levels were correlated with plasma 8-iso-PG F2 α levels. Furthermore, it was shown that the order parameter (S) of RBCs was correlated with plasma total homocysteine and plasma 8-iso-PG F2 α levels, indicating that reduced membrane fluidity of RBCs was associated with hyperhomocysteinemia and increased oxidative stress. Multivariate regression analysis also demonstrated that, after adjustment for general risk factors, plasma total homocysteine and plasma 8-iso-PG F2 α were independent determinants of membrane fluidity of RBCs, respectively. Because the deformability of RBCs might be highly dependent on the membrane fluidity [15,16], the reduction in membrane fluidity of RBCs could cause a disturbance in the blood rheologic behavior and the microcirculation in hypertension.

It was shown that shear rate, shear stress and blood viscosity were correlated with membrane fluidity of RBCs [24]. The finding proposed that *in vivo* shear forces

might participate in the control of RBC membrane fluidity, and that RBCs might adapt their membrane properties to blood flow conditions. It was also demonstrated that RBC membranes might become more rigid after myocardial infarction, which could contribute to the decreased RBC deformability and the increased blood viscosity in this group of patients [25]. On the other hand, Cazzola *et al.* [26] reported that the membrane fluidity of RBCs was decreased in the obese subjects, and proposed that a decrease in RBC membrane fluidity could contribute to a reduction of the rate of blood flow and the oxygen diffusion through the RBC membranes and its exchange with tissues. It might be possible that alterations in RBC membrane fluidity with hyperhomocysteinemia and increased oxidative stress would be strongly linked to the progression of circulatory disorders.

The precise mechanisms by which hyperhomocysteinemia and oxidative stress could affect the membrane functions are not fully understood. Wang *et al.* [27] showed a significant association between serum homocysteine and oxidative stress in elderly patients with obstructive sleep apnea syndrome. It was also demonstrated that homocysteine increased the oxidative stress levels dose- and time-dependently in cultured bovine retinal endothelial cells [28]. These findings might be consistent with our present finding indicating that plasma total homocysteine levels were correlated with plasma 8-iso-PG F2 α levels. On the other hand, it was shown that endothelium-dependent vasodilation was impaired in subjects with hyperhomocysteinemia and elevated oxidative stress levels [4,6,29,30]. Stühlinger *et al.* [31] demonstrated that homocysteine might directly impair the NO synthase pathway *in vitro*. In a study presented earlier, we showed that an NO donor significantly improved membrane fluidity of RBCs in hypertensive subjects, suggesting that NO could have a beneficial effect on the rheologic behavior of RBCs and the microcirculation in hypertension [18]. In the present study, it was demonstrated that decreased plasma NO-metabolite levels were associated with not only reduced membrane fluidity of RBCs, but also hyperhomocysteinemia and increased 8-iso-PG F2 α levels. One hypothesis is that the effects of homocysteine and oxidative stress on membrane fluidity of RBCs might be mediated, at least in part, by reducing NO-bioavailability, although direct actions of homocysteine and oxidative stress on membrane structural and functional properties cannot be fully excluded [32,33]. Further studies should be performed to assess more precisely the interactions among homocysteine, oxidative stress and endothelial function, and their role in the regulation of membrane functions in hypertension.

5. Conclusion

The results of the present study demonstrated that both

plasma total homocysteine and 8-iso-PG F2 α levels were elevated in hypertensive men compared with normotensive men. Furthermore, it was shown that reduced membrane fluidity of RBCs was correlated with increased plasma total homocysteine and 8-iso-PG F2 α levels, and decreased plasma NO metabolite levels, suggesting that abnormalities in RBC membranes in hypertension might be associated with hyperhomocysteinemia, increased oxidative stress, and endothelial dysfunction. Although this is a cross-sectional and correlative study in Japanese men, the results of the present study could provide a hypothesis that hyperhomocysteinemia and oxidative stress with endothelial dysfunction might have a close correlation with the impaired rheologic behavior of RBCs and the microcirculation, and contribute, at least in part, to the circulatory disorders in hypertensive men.

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