Influences of Increased Oxidative Stress on Endothelial Function, Platelets Function, and Fibrinolysis in Hypertension Associated with Glucose Intolerance

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The effect of oxidative stress on endothelial function, platelet function, and fibrinolysis in hypertension with or without glucose intolerance was examined. The endothelium, platelets and fibrinolysis play important roles in the progression of atherosclerosis and interact with each other. We have previously demonstrated that glucose intolerance impairs endothelial function in hypertension, but its precise mechanisms have not been clarified. Hypertensive patients were divided by the results of 75-g oral glucose tolerance test into a normal glucose metabolism group (n=65) and a glucose intolerance group (n=47). The plasma level of thiobarbituric acid-reactive substances (TBARS) was assessed as a marker of oxidative stress. Endothelial function was assessed by flow-mediated dilatation (FMD), platelet function by the concentration of ADP dose inducing half-maximal aggregation (EC50), and fibrinolytic parameters by radioimmunoassay. These functions were assessed before and after acute administration of vitamin C. FMD was reduced while TBARS and fibrinolytic parameters were higher in patients with glucose intolerance than in those with a normal glucose metabolism. Vitamin C increased FMD and reduced fibrinolytic parameters significantly in the glucose intolerance group, but not in the group with normal glucose metabolism. On the other hand, the EC50 was similar in both groups. In conclusion, glucose intolerance aggravates oxidative stress, thereby contributing to the impairment of endothelial function in patients with hypertension. These abnormalities affect fibrinolysis but not platelet function. (Hypertens Res 2003; 26: 295–300)

Key Words: endothelium, hemostasis, fibrinolysis, insulin resistance, oxidative stress

Introduction

Patients with either hypertension or diabetes mellitus present abnormalities of endothelial function, platelet function and fibrinolysis (1, 2). These abnormalities are thought to play crucial roles in the progression of atherosclerosis and to contribute to the occurrence of cardiovascular accidents (3). Hypertension is frequently accompanied by an abnormal glucose metabolism (4). We previously demonstrated that glucose intolerance as assessed by the 75-g oral glucose tolerance test (OGTT) contributes to the impairment of endothelial function observed in hypertensive patients (5). However, it has not been clarified whether glucose intolerance also affects platelet function and/or fibrinolysis.

Increased oxidative stress is considered to contribute to vascular damage (6, 7), and several studies have demonstrated that increased oxidative stress impairs endothelial function by reducing nitric oxide (NO) availability (6, 7). Gopaul et al. (8) demonstrated increased oxidative stress even in the early stage of abnormal glucose metabolism, namely, in patients showing glucose intolerance. We there-
fore hypothesized that increased oxidative stress contributed to the more marked endothelial dysfunction observed in hypertensive patients with glucose intolerance. Since NO has been shown to inhibit platelet aggregation (9), increased oxidative stress could induce platelet dysfunction via the inactivation of NO. In addition, it has been well documented that the endothelium interacts with both platelets and the process of fibrinolysis (9, 10), and it is possible that endothelial dysfunction affects platelet function and/or fibrinolysis (3). However, no study has systematically evaluated the state of oxidative stress in hypertensive patients with glucose intolerance, or the effect of oxidative stress on endothelial function, platelet function, and fibrinolysis in such cases.

The present study was conducted to confirm the following hypotheses: 1) Glucose intolerance increases oxidative stress and this increase contributes to the more marked endothelial dysfunction observed in hypertension; 2) These abnormalities affect fibrinolysis and the function of platelets.

Methods

Patients and Study Protocol

We studied 118 consecutive ambulatory Japanese patients with mild-to-moderate hypertension and without any other serious medical problems requiring specific treatments, who visited the Ichihara Hospital of the Teikyo University School of Medicine. Prior to their participation in the present study, all the patients were instructed to modify their lifestyle for about 3 months. Inclusion criteria were as follows: no history of treatment with antihypertensive drugs, fasting plasma glucose < 7.0 mmol/l, and an average systolic/diastolic blood pressure of < 140/90 mmHg in the three consecutive visits prior to the study. Patients with secondary hypertension were excluded. Then, approximately 3 months after lifestyle modification, 75-g OGTT was performed, and the patients were divided into two groups according to the 2-h blood glucose level: those with a blood glucose concentration of < 7.8 mmol/l were allocated to the normal glucose metabolism (NGM) group, and those with ≥ 7.8 mmol/l to the glucose intolerance (GIT) group; 6 patients with ≥ 11.1 mmol/l of blood glucose were excluded with the diagnosis of diabetes mellitus. Thus, 112 patients (60 men, 52 women; 54 ± 9 years of age) were entered into this study. The Ethical Committee of Ichihara Hospital, Teikyo University School of Medicine, approved the study protocol. Written informed consent was obtained from all patients. All procedures were in accordance with our institutional guidelines. Each patient underwent blood tests and assessments of endothelial and platelet functions before and after vitamin C infusion.

Vitamin C Infusion

At 7:30 AM, a plastic needle was placed in the antecubital vein of the left forearm for blood sampling, and another plastic needle was placed in the antecubital vein of the left pedicle of the lower leg and used for intravenous infusion of normal saline at a rate of 20 ml/h. Thirty min later (8:00 AM), blood was drawn from the left arm and an endothelial function test was performed. Then, the patient was given a bolus injection of vitamin C (at 15 mg/kg; Ascoltin, Tanabe Co., Ltd., Tokyo, Japan) followed by constant vitamin C infusion at a rate of 2.0 g/h for 1 h via the needle placed in the left lower leg. At 9:00 AM, blood was again collected, and the endothelial function test was repeated.

Endothelial Function Test

The ultrasonographic brachial endothelial function test has been described previously (5). Briefly, an ultrasonographic system (SSH-160A; Toshiba, Tokyo, Japan) equipped with a 7.5-MHz transducer was used to image the brachial artery of the right arm. In the long-axis view, a straight segment (at least 1 cm) of the brachial artery immediately above the antecubital fossa was used. M-mode tracings were obtained together with simultaneous electrocardiographic recordings using a strip-chart recorder. After that, the cuff was inflated at the upper arm to 20 mmHg above systolic blood pressure for 5 min, and deflated rapidly. The diameter of the brachial artery was measured at 60–70 s after deflation.

Endothelial function assessed by flow-mediated dilatation (FMD) of the brachial artery was calculated by the following formula: (diameter of brachial artery after hyperemia - diameter of brachial artery before hyperemia) / 100 / diameter of brachial artery before hyperemia.

Platelet Function

Platelet function was assessed using a Born’s aggregometer (HEMA tracer IV; NBS, Tokyo, Japan). Nine ml of blood was collected in a syringe containing 1 ml of 3.8% sodium citrate. The whole blood was centrifuged at 600 rpm for 10 min to obtain platelet-rich plasma. The remaining blood was again centrifuged at 3,000 rpm for 10 min to obtain platelet-poor plasma. Both plasma samples were transferred to polystyrene tubes. The extent of aggregation was expressed as a percent of the baseline. Light transmission of platelet-poor plasma was set at 100% and untreated platelet-rich plasma at 0%. Adenosine 5′-diphosphate (ADP) (MCM Medica; Tokyo, Japan) was used as an aggregating reagent and the EC50 value (i.e., the concentration of agonist required to induce half-maximal aggregation) was determined (11). The EC50 for ADP was determined by a dose-response assay that measured the extent of aggregation. Seven different concentrations of ADP (100, 40, 30, 20, 15, 10 and 5 µmol/l) were investigated.
Table 1. Clinical Characteristics of Hypertensive Patients with Glucose Intolerance and of Those with Normal Glucose Metabolism

<table>
<thead>
<tr>
<th></th>
<th>GIT (n = 47)</th>
<th>NGM (n = 65)</th>
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<tbody>
<tr>
<td>Age (years)</td>
<td>54 ± 10</td>
<td>55 ± 8</td>
</tr>
<tr>
<td>Male/female</td>
<td>31/16</td>
<td>40/25</td>
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<tr>
<td>Smokers</td>
<td>8</td>
<td>10</td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>154 ± 15</td>
<td>152 ± 14</td>
</tr>
<tr>
<td>DBP (mmHg)</td>
<td>95 ± 11</td>
<td>93 ± 10</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>25 ± 3</td>
<td>24 ± 2</td>
</tr>
<tr>
<td>TC (mmol/l)</td>
<td>5.9 ± 1.1</td>
<td>5.6 ± 0.9</td>
</tr>
<tr>
<td>TG (mmol/l)</td>
<td>1.9 ± 1.0**</td>
<td>1.3 ± 0.5</td>
</tr>
<tr>
<td>HDL (mmol/l)</td>
<td>1.4 ± 0.5*</td>
<td>1.6 ± 0.6</td>
</tr>
<tr>
<td>FINS (pmol/l)</td>
<td>6.0 ± 0.9**</td>
<td>5.4 ± 0.4</td>
</tr>
<tr>
<td>FIB (g/l)</td>
<td>46 ± 23**</td>
<td>38 ± 19</td>
</tr>
<tr>
<td>FDP (µg/ml)</td>
<td>2.4 ± 0.5</td>
<td>2.3 ± 0.6</td>
</tr>
</tbody>
</table>

GIT, patients with glucose intolerance; NGM, patients with normal glucose metabolism; smokers, number of smokers; SBP, systolic blood pressure; DBP, diastolic blood pressure; BMI, body mass index; TC, total cholesterol; TG, triglycerides; HDL, high-density lipoprotein cholesterol; FIB, fasting blood glucose; FINS, fasting plasma insulin concentration; FIB, plasma fibrinogen level; FDP, fibrinogen/fibrin degradation products. *p < 0.05 and **p < 0.01 vs. patients with normal glucose metabolism.

Measurement of Oxidative Stress

As a marker of oxidative stress, lipid peroxidation products in plasma samples were measured as thiobarbituric acid-reactive substances (TBARS). Plasma was mixed with 2% butylated hydroxytoluene and quinlanilla reagent (26 mmol/l thiobarbituric acid and 918 mmol/l trichloroacetic acid). The mixture reaction was boiled for 15 min. Thereafter, the reaction mixture was cooled and centrifuged at 3,000 g for 10 min. The soluble phase was measured with a spectrophotometer at a wavelength of 535 nm (J2). The co-efficient of variation in the measurement was 3.8%.

Laboratory Measurements

Blood glucose level was determined using a Glucoroder SX Analyzer (A&T, Kyoto, Japan). Plasma triglycerides, total cholesterol, and high-density lipoprotein cholesterol were measured enzymatically using a Hitachi 731 Analyzer (Tokyo, Japan). Plasma insulin level was determined by radioimmunoassay (SRL, Tokyo, Japan). Vitamin C concentration in metaphosphoric acid-precipitated plasma samples was measured by high-performance liquid chromatography with electrochemical detection. Tissue plasminogen activator antigen (tPA) (Imulize tPA kit; Biopool, Sweden) and tissue plasminogen activator inhibitor-1 antigen (PAI-1) (PAI-1 ELISA kit; Monozyme, Ibaraki, Japan) were determined by radioimmunoassay. Fibrinogen was determined with a one-stage clotting assay kit (Thrombocheck Fib; International Reagents Corp., Freiberg, Germany). Fibrinogen/fibrin degradation products were measured by a latex photometric immunoassay method (COBAS MIRA, Tokyo, Japan). For the determinations of cyclic guanosine 3'5' monophosphate (cyclic GMP) level in platelets (J2), we used the platelet-rich plasma samples. After incubation with test compounds, the reaction was stopped by adding 10% perchloric acid, and the supernatant was assayed for cyclic GMP level using a radioimmunoassay kit (Yamasa, Noda, Japan).

Statistical Analysis

Data are expressed as the mean ± SD. Statistical analysis was performed using the SPSS software package (SPSS Inc., Chicago, USA). Within-group differences were evaluated by paired t-test. Comparisons between groups were made using the independent sample t-test with Levene’s test for equality of variance. In the assessment of the difference of the effect of vitamin C, the delta changes in FMD, EC50 for ADP, tPA, PAI-1, and TBARS were compared between GIT and NGM using the independent sample t-test with Levene’s test. Values of p < 0.05 were considered to indicate statistical significance.

Results

Table 1 summarizes the clinical characteristics of patients in the GIT and NGM groups. Total cholesterol, triglycerides, fasting blood sugar, and fasting plasma insulin were higher in the GIT than in the NGM group, while the reverse was true of high-density lipoprotein (HDL) cholesterol.

Table 2 shows the parameters of endothelial function, platelet function, fibrinolysis, and oxidative stress in hypertensive patients with GIT and in those with NGM before and after the administration of vitamin C. FMD was lower while tPA antigen, PAI-1 antigen and TBARS were higher in the GIT than in the NGM group. The EC50 for ADP, intraplatelets cyclic GMP levels, and plasma level of vitamin C were similar in both groups. Vitamin C infusion increased FMD and decreased tPA and TBARS in the GIT group, but not in the NGM group. Vitamin C infusion decreased the EC50 for ADP and decreased intra-platelet cyclic GMP levels to similar levels in both groups.

The delta changes in endothelial function, platelet function, intra-platelet cyclic GMP levels, tPA, PAI-1, and TBARS in GIT and NGM are illustrated in Fig. 1. The changes in endothelial function and tPA differed significantly between GIT and NGM.

Table 3 shows the correlation coefficients of the linear regression analysis of the parameters of endothelial function, hemostatic variables and oxidative stress before the administration of vitamin C and those of their changes by the administration of vitamin C. TBARS correlated with FMD and tPA, but not with EC50 for ADP. FMD correlated with tPA but not
with EC50 for ADP. Before and after the administration of vitamin C, the delta change in tPA correlated with that in FMD.

**Discussion**

**Oxidative Stress and Endothelial Function**

Experimental studies have demonstrated that the increased oxidative stress observed in hypertension is generated by several mechanisms, namely, reduced form of nicotinamide-adenine dinucleotide (phosphate) (NAD(P)H) oxidase, xanthine oxidase, endothelial nitric oxide synthetase, and carbonyl proteins (6, 7, 13). Some clinical studies have demonstrated an improvement of endothelial function after vitamin C infusion, and they suggested that oxidative stress was increased in patients with hypertension (14, 15). However, these studies did not investigate the status of glucose metabolism. It is well known that patients with hypertension frequently (in about 30–40% of cases) have a latent abnormal glucose metabolism, as assessed by OGTT (16, 17). TBARS, a marker of oxidative stress (18), was higher in the GIT than in the NGM group. In addition, acute administration of vitamin C improved endothelial dysfunction in GIT but not in NGM subjects. The same dose of vitamin C was shown to have improved endothelial function in patients with acute hyperinsulinemia, in patients with abnormal glucose metabolism, and in patients with coronary heart disease (19–21).

Jackson et al. (22) demonstrated that a high physiological concentration of vitamin C (>150 µmol/l) prevented the interaction of NO and superoxide, and in the present study the
blood concentration of vitamin C was well within this range. In patients with an abnormal glucose metabolism, hyperglycemia directly increases the production of oxygen radicals and hyperinsulinemia also attenuates endothelial function via increased oxidative stress caused by the activation of NAD(P)H oxidase (13, 19, 23). Therefore, glucose intolerance aggravates oxidative stress, and this aggravation might contribute to the more marked endothelial dysfunction observed in hypertensive patients with glucose intolerance.

**Platelet Function**

In the present study, while endothelial function was impaired and TBars was higher in GIT than in NGM subjects, the EC$_{50}$ of ADP was similar in both groups. Vitamin C improved platelet function in the patients irrespective of the improvement of endothelial function. In addition, the EC$_{50}$ of ADP did not correlate with the extent of FMD. Thus, while we observed an interaction between the endothelium and platelets, these results suggest that in the early stages endothelial abnormality is independent of platelet function, and that oxidative stress does not have a universal effect on endothelial or platelet functions.

Because we did not measure intra-platelet oxidative stress, we were unable to determine the precise reason why the increase in the plasma level of oxidative stress did not affect the function of platelets. As for the lack of universal influence of oxidative stress on the endothelium and platelets, we think that while increased oxidative stress has been suggested to enhance the aggregability of platelets in patients with overt diabetes mellitus (24), the increase of oxidative stress in GIT is not high enough to affect platelet function. Moreover, the extent of oxidative stress differs in various tissues (25). Seghieri et al. (26) proposed that an increased intra-platelet vitamin C level counteracts the oxidative stress in platelets. In the present study, the intra-platelet oxidative stress might have been effectively reduced by the intra-platelet antioxidant, causing the level to be lower than that in plasma. In both the GIT and NGM groups, vitamin C increased the EC$_{50}$ of ADP and intra-platelet cyclic GMP, which is the second messenger of NO (27). We confirmed that the EC$_{50}$ of ADP did not change at 8:00 AM and 9:00 AM without the administration of vitamin C in 8 hypertensive subjects. Thus the circadian variation of platelet function might not have affected the present results. The present results suggested that a massive amount of vitamin C might reduce the basal redox state and the aggregability of platelets. In fact, oral administration of a massive dose (2 g) of vitamin C has been shown to reduce platelet aggregability in normal volunteers (28).

**Fibrinolysis**

Under hemostatic balance, platelets, the coagulation system and fibrinolysis each play important roles, and each influence each other (3). In the present study, the levels of tPA antigen and PAI-1 antigen were higher in GIT than in NGM subjects. FMD correlated with tPA, but not with the EC$_{50}$ for ADP, fibrinogen or fibrinogen/fibrin degradation products. Upon administration of vitamin C, parallel changes of endothelial function and the level of tPA were observed. It was thus confirmed that there is a close relationship between endothelial function and fibrinolysis in patients with hypertension, and this relationship did not depend on the function of platelets or the coagulation system. Endothelial dysfunction, rather than increased oxidative stress, may be the more potent contributing factor for fibrinolysis.

**Clinical Implications and Limitations**

It is well understood that the assessment of the presence/absence of atherosclerotic risk factors is crucial in the evaluation of vascular damage in patients with hypertension (1, 2). The present results suggest that the assessment of oxidative state is an additional risk marker of vascular damage. Recent studies have reported other useful markers of oxidative stress, namely, 8-isoprostaglandin, plasma anti-oxi-

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**Table 3. Correlation Coefficients of the Linear Regression Analysis of Parameters of Endothelial Function, Hemostatic Variables and Oxidative Stress before the Administration of Vitamin C and Those of Their Changes by the Administration of Vitamin C**

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<tr>
<th></th>
<th>FMD</th>
<th>PLT</th>
<th>tPA</th>
<th>PAI-1</th>
<th>FIB</th>
<th>TBars</th>
</tr>
</thead>
<tbody>
<tr>
<td>FMD</td>
<td></td>
<td></td>
<td>- 0.37*</td>
<td>- 0.19*</td>
<td>- 0.36*</td>
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<tr>
<td>PLT</td>
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<td>tPA</td>
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<tr>
<td>PAI-1</td>
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<td>FIB</td>
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<td>TBars</td>
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<tr>
<th></th>
<th>delFMD</th>
<th>delPLT</th>
<th>deltPA</th>
<th>delPAI-1</th>
<th>delTBARS</th>
</tr>
</thead>
<tbody>
<tr>
<td>delFMD</td>
<td></td>
<td></td>
<td>- 0.34**</td>
<td></td>
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<tr>
<td>delPLT</td>
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<td>deltPA</td>
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<tr>
<td>delPAI-1</td>
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<td>delTBARS</td>
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FMD, flow-mediated vasodilatation of brachial artery; PLT, the concentration of ADP required to induce half-maximal aggregation of platelets; tPA, tissue plasminogen activator antigen; PAI-1, plasminogen activator inhibitor-1 antigen; FIB, plasma fibrinogen level; TBars, plasma level of thiobarbituric acid-reactive substances; deltaFMD, delta changes in FMD by the administration of vitamin C; delPLT, delta changes in PLT by the administration of vitamin C; deltPA, delta changes in tPA by the administration of vitamin C; delPAI-1, delta changes in PAI-1 by the administration of vitamin C; delTBars, delta changes in TBars by the administration of vitamin C. * $p < 0.05$, ** $p < 0.01$. 

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dant capacity, hydrogen peroxide production in leukocytes or in platelets, and so on (18). Further studies will be needed to investigate the oxidative state and its efficacy as a marker of vascular damage in patients with hypertension with or without GIT, as well as to compare the oxidative state and its clinical significance between patients with hypertension without GIT and normotensives with normal glucose metabolism. The amount of ADP required for platelet aggregation is one of the parameters used to assess platelet function (11). However, this parameter is an indicator of intrinsic platelet function. Further evaluation of the interaction between the endothelium and platelets under rheological dynamic conditions will be needed.

In conclusion, glucose intolerance aggravates oxidative stress in patients with mild-to-moderate hypertension. This high level of oxidative stress contributes to the marked impairment of endothelial function observed in hypertensive patients. These abnormalities affect fibrinolysis but not platelet function.

References