Calcium Channel Blocker, Azelnidipine, Reduces Lipid Hydroperoxides in Patients with Type 2 Diabetes Independent of Blood Pressure

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Abstract. Anti-hypertensive agents with antioxidative effects are potentially useful for diabetic patients with hypertension to prevent the onset and progression of their complication. While dihydropyridine-type calcium antagonists are among the frequently used anti-hypertensive drugs, azelnidipine, a novel calcium antagonist, has been reported to have a unique anti-oxidative effect in vitro and in animals. In this study, we measured lipid hydroperoxides in human sample using diphenyl-1-pyrenylphosphine for the first time, and used the value of lipid hydroperoxides as an index of oxidative stress. Then, we compared the antioxidative properties of azelnidipine and amlodipine, a frequently used calcium antagonist in hypertensive diabetic patients. Administration of vitamin C and E for 8 weeks significantly reduced lipid hydroperoxides in erythrocyte membrane in normal subjects. In hypertensive diabetic patients, azelnidipine treatment for 12 weeks induced a more significant fall in erythrocyte lipid hydroperoxide level than amlodipine, though blood pressure during each treatment was comparable. Our data confirm the usefulness of lipid hydroperoxides in erythrocyte membrane as a marker of oxidative stress in vivo, and indicate that azelnidipine has a unique antioxidative property in human.

Key words: Oxidative stress, Lipid hydroperoxides, Diphenyl-1-pyrenylphosphine (DPPP), Diabetes mellitus, Azelnidipine, Hypertension

OXIDATIVE stress impairs various cellular functions and is one of the important pathological mechanisms of many diseases. In diabetes mellitus, oxidative stress plays important roles in the progression of diabetic complications. In particular, type 2 diabetics with hypertension are at high risk of atherosclerosis and vascular complications which are associated with the increase of oxidative stress, but optimal control of blood pressure substantially reduces excess cardiovascular risks in patients with diabetes with the reduction of oxidative stress [1, 2].

Several types of anti-hypertensive drugs are currently available for the treatment of hypertension. Dihydropyridine-type calcium channel blockers (DCCB) are one group of the most commonly used anti-hypertensive agents. Several studies have demonstrated in a variety of cellular and subcellular preparations that DCCB has dose-dependent antioxidant properties [3–6]. These agents are considered beneficial in the treatment of diseases associated with oxidative stress such as atherosclerosis [7–11]. Amlodipine, a commonly used DCCB, has been shown also to have potent antioxidant effects via its membrane physico-chemical interactions, independent of calcium channel modulation [12].

Azelnidipine is a relatively new DCCB with long-
acting anti-hypertensive action and few incidences of tachycardia. Because this agent is highly lipid soluble, it is retained in the vascular wall [13]. In addition, it has more potent antioxidant effects at its clinical dose in cultured human arterial endothelial cells than other DCCBs such as amlodipine [14]. To our knowledge, however, there are no reports of the antioxidative effects of azelnidipine in a clinical setting.

Diphenyl-1-pyrenylphosphine (DPPP) reacts with hydroperoxides stoichiometrically to yield a fluorescent compound, DPPP-oxide, and hydroxide. Due to its lipophilic nature and high fluorescence intensity, it can be applied for quantitative analysis of lipid peroxidation [15]. While DPPP has been frequently used to measure lipid hydroperoxides in an HPLC post-column system [16–19], it can also be applied to measure the extent of oxidation in a solution [20] and also in low-density lipoprotein particles [21]. Furthermore, DPPP has been applied as a fluorescent probe for lipid peroxidation in cultured cells [15, 22, 23] and intact organs [24].

In the present study, for the first time, we applied the fluorescent derivative DPPP as a sensitive and selective probe to measure lipid hydroperoxide in human plasma and erythrocyte membrane and compared the antioxidative properties of azelnidipine with those of amlodipine in type 2 diabetic patients with hypertension. The results demonstrated that the DPPP probe is useful for measurement of lipid hydroperoxide in human erythrocyte membrane as a marker of oxidative stress, and that azelnidipine has unique antioxidative effects compared with amlodipine.

Materials and Methods

Subjects

To evaluate the effects of vitamins C and E on the value of lipid hydroperoxides with DPPP, healthy volunteers were asked to participate in the study. The inclusion criteria of this study were subjects who were not diagnosed with any diseases and not taking any medications. To evaluate the effects of azelnidipine, patients with type 2 diabetes mellitus who visited Juntendo University Hospital (Tokyo, Japan) from April 2005 to August 2005 were asked to participate in this study. The inclusion criteria of this study were patients taking amlodipine (5 mg/day) for at least six months. The diagnosis of type 2 diabetes was based on the current WHO criteria. Patients with diabetic microangiopathy, severe renal or hepatic disease, overt cardiovascular disease, and malignancy were excluded. Furthermore, patients with more than 2% variation of HbA1c value within six months were excluded. None of the subjects had taken vitamin C, vitamin E, or probucol, which are known antioxidant compounds. The hospital ethics committee approved this study protocol and informed consent was obtained from each subject.

Study protocol

To evaluate the effect of vitamin C and vitamin E on the value of lipid hydroperoxides with DPPP, we measured DPPP-oxide in 15 healthy volunteers before and 8 weeks after taking antioxidant supplement, vitamin C (1000 mg/day) and vitamin E (300 mg/day). During the study period, all subjects were prohibited from taking any medication.

The effect of azelnidipine (16 mg/day) was evaluated in 14 type 2 diabetic patients with hypertension. Fasting blood samples were obtained at baseline during treatment with amlodipine (5 mg/day). Then, amlodipine (5 mg/day) was changed to azelnidipine (16 mg/day). Each patient was reviewed once a month for evaluation of general health and compliance with medication. The doses of every medication including anti-diabetic drugs were not changed during the study. Twelve weeks after changing amlodipine (5 mg/day) to azelnidipine (16 mg/day), fasting blood samples were obtained again for analysis.

Lipid extraction

Approximately 5 ml blood was drawn into EDTA-2Na test tube and centrifuged at 1000 × g for 15 min at 4°C. After separation of plasma and removal of the buffy coat, the erythrocytes were washed three times with isotonic saline. The packed erythrocytes and separated plasma obtained were stored at –70°C until use. One hundred microliters of packed erythrocytes in 1 ml of 5 mM ammonium-acetate buffer (pH 7.4) were centrifuged at 40,000 × g for 15 min and the supernatant was removed. The procedure was repeated three times for preparation of erythrocyte ghost. The lipid was then extracted from white ghost and from plasma by the method of Bligh and Dyer [25] with minor modifi-
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Special care was taken during sample processing to avoid artifactual oxidation (dim light was used throughout the analysis, contact with air was minimized, and all samples were stored under argon). In brief, the sample (resulting white ghost or 100 µl of plasma) was mixed with 0.7 ml water (containing acetic acid with final concentration of 0.1 M), 2 ml methanol [containing 0.003% butylated hydroxytoluene (BHT)] and 1 ml chloroform. After 30-min incubation at room temperature under constant stirring, 1 ml chloroform and 1 ml water were added. The samples were mixed, and then centrifuged at 1500 × g for 15 min. The supernatant was removed, then the resultant chloroform layer was collected and evaporated under reduced pressure at 20°C. The residue was dissolved in 1 ml of mixture of methanol and chloroform (1 : 2), which contained 0.003% BHT, and stored at −70°C until measurement.

Determination of total lipid hydroperoxides chemicals

DPPP was purchased from Dojindo Laboratories (Kumamoto, Japan). To a screw-cap test tube, we added 10 µl aliquot of lipid solution and 50 µl of DPPP solution [1 mg/10 ml in methanol-chloroform (1 : 1)]. The tube was placed in a dry bath at 60°C for 60 min in the dark. After cooling on an ice bath, 3 ml of methanol was added to the mixture. After shaking, the fluorescence intensity was measured at 380 nm (excitation at 352 nm) using a fluorometer (model F-4500 fluorospectrophotometer, Hitachi, Tokyo, Japan) and the intensity of fluorescence per lipid weight was calculated. To confirm reproducibility, measurements were repeated four times for both plasma and erythrocytes obtained from the same donor at different times. The distribution of lipid hydroperoxides concentrations showed satisfactory and no substantial change.

Measurement of carboxy-methyl lysine (CML), malonaldehyde-modified low-density lipoprotein (MDA-LDL) and other laboratory assays

Plasma glucose concentrations were determined with a glucose oxidase method (Kainos, Tokyo). Glycosylated hemoglobin (HbA1c) was measured by high performance liquid chromatography with a normal range of 4.3% to 5.8% (Tohsoh, Tokyo). Total-cholesterol, HDL-cholesterol and triglycerides levels were measured using standard enzymatic methods (Kainos, Tokyo) and LDL-cholesterol values were calculated using Friedewald’s formula. Plasma CML and MDA-LDL levels were measured by ELISA (enzyme-linked immunosorbent assay) based on the principle reported previously [26–28]. These assays were performed by hospital personnel in the clinical chemistry department except for CML and MDA-LDL, which were measured in a commercial laboratory (SRL, Tokyo).

Statistical analysis

Values were presented as mean±SD. Wilcoxon T-test was used to compare differences between baseline and follow-up measures within groups. Significance was defined as P<0.05.

Results

Administration of vitamins C and E reduces lipid hydroxide in erythrocyte membrane of healthy subjects

The clinical characteristics of the healthy subjects are summarized in Table 1. First, we validated the measurement of lipid hydroperoxides using DPPP in these subjects. Whereas treatment with vitamin C and vitamin E for 8 weeks did not affect most of the laboratory data investigated, the same treatment significantly reduced CML level. Furthermore, the same treatment did not alter the level of lipid hydroperoxide in plasma but significantly decreased the level in erythrocytes (Table 2). These results suggest that measurement of

| Table 1. Clinical characteristics of participating subjects |
|-----------|----------------|----------------|
| N          | Healthy volunteers | Type 2 diabetes |
| Age (years) | 32.6 ± 4.8         | 62.35 ± 9.8     |
| Sex (male/female) | 11/4               | 7/7             |
| BMI (kg/m²)  | 21.7 ± 2.5         | 26.4            |
| Duration of diabetes (years) | —                  | 12.6 ± 4.6      |
| Complications (n) (non/neuropathy/retinopathy/nephropathy) | —                  | 5/3/5/7        |
| Therapy (n) (diet/OHA/insulin) | —                  | 2/10/2         |

Data are expressed as mean ± SD. BMI, Body mass index.
Azelnidipine reduces lipid hydroperoxide in erythrocyte membrane in hypertensive type 2 diabetics

To investigate the antioxidative capacity of azelnidipine relative to amlodipine, we recruited patients with type 2 diabetes treated with amlodipine. By changing amlodipine to azelnidipine, we compared the effect of each drug. The patients’ characteristics are summarized in Table 3. Plasma glucose, HbA1c, total-cholesterol, high density lipoprotein (HDL)-cholesterol, low-density lipoprotein (LDL)-cholesterol, triglyceride and arterial blood pressure were not different under the two treatments. Furthermore, the levels of MDA-LDL and CML, which are oxidative stress markers, were comparable under the two treatments. However, lipid hydroperoxide level in erythrocytes under azelnidipine treatment was significantly lower (by about 18%) than under amlodipine (p<0.05, Table 3). These results emphasize the potent antioxidative capacity of azelnidipine in clinical setting compared with amlodipine.

Discussion

To our knowledge, this is the first report using DPPP for evaluation of lipid hydroperoxides to assess one aspect of oxidative stress in a clinical setting. Our results demonstrated that the level of lipid hydroperoxides in erythrocytes of healthy subjects was decreased following administration of vitamins C and E, suggesting that this method reflects at least one aspect of oxidative stress in human. In addition, we tried to assess the antioxidative property of azelnidipine, a DCCB reported to have unique antioxidative effects \[14, 29\], and in animals \[30, 31\], but not yet in human samples. While blood pressure during treatment with azelnidipine and amlodipine was comparable, azelnidipine decreased lipid hydroperoxides in erythrocyte membrane compared with amlodipine. As far as we know, this is also the first clinical study that identifies the antioxidative property of azelnidipine.

In this study, we selected the fluorescent derivative DPPP as a sensitive and selective probe for measuring lipid hydroperoxides in human plasma. Based on our method of using DPPP in this study, the precise origin of lipid hydroperoxide that reacted with DPPP is not clear. It was reported that most of the lipid hydroperoxides in biological tissues were phosphatidylcholine hydroperoxide and phosphatidylethanolamine hydroperoxide \[32, 33\]. In addition, very small amounts of cholesteryl ester hydroperoxide and minute amounts of triglyceride hydroperoxides in plasma of healthy adults were reported \[34–36\]. Since the reactions of DPPP are selective to hydroperoxides of fatty acid and glycerides but not to di-alkylperoxide \[15\], the majority of lipid hydroperoxides detected in our assay may be phosphatidyl lipid hydroperoxide. In any case, consid-

### Table 2. Effect of taking vitamin C and E in healthy volunteer

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>After vitamins C and E</th>
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<tbody>
<tr>
<td>Fasting blood sugar (mg/dl)</td>
<td>92 ± 6.6</td>
<td>103 ± 13.3</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>4.7 ± 0.2</td>
<td>4.6 ± 0.2</td>
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<tr>
<td>Total cholesterol (mg/dl)</td>
<td>184.8 ± 27.5</td>
<td>198.6 ± 21.5</td>
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<tr>
<td>High-density lipoprotein (mg/dl)</td>
<td>64.8 ± 12.4</td>
<td>67.1 ± 14.0</td>
</tr>
<tr>
<td>Low-density lipoprotein (mg/dl)</td>
<td>102.5 ± 23.1</td>
<td>110.1 ± 21.1</td>
</tr>
<tr>
<td>Triglyceride (mg/dl)</td>
<td>107.7 ± 64.4</td>
<td>118.7 ± 54.5</td>
</tr>
<tr>
<td>MDA-LDL (IU/L)</td>
<td>88.7 ± 32.2</td>
<td>93.2 ± 26.6</td>
</tr>
<tr>
<td>N-ε(carboxymethyl) lysine (µg/ml)</td>
<td>6.81 ± 1.33</td>
<td>5.67 ± 1.15*</td>
</tr>
<tr>
<td>Lipid hydroperoxide (Fluorescence intensity/lipid)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasma</td>
<td>0.326 ± 0.15</td>
<td>0.292 ± 0.18</td>
</tr>
<tr>
<td>Erythrocytes</td>
<td>1.27 ± 0.29</td>
<td>1.00 ± 0.32*</td>
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</table>

Data are expressed as mean ± SD.  
* P<0.05 vs. baseline.

MDA-LDL, malondialdehyde-modified low density lipoprotein

### Table 3. Comparison of the effect of amlodipine and azelnidipine.

<table>
<thead>
<tr>
<th></th>
<th>Amlodipine</th>
<th>Azelnidipine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fasting blood sugar (mg/dl)</td>
<td>149 ± 48</td>
<td>139 ± 54</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>6.8 ± 0.6</td>
<td>6.8 ± 0.7</td>
</tr>
<tr>
<td>Total cholesterol (mg/dl)</td>
<td>178.5 ± 27.2</td>
<td>181.8 ± 26.8</td>
</tr>
<tr>
<td>High-density lipoprotein (mg/dl)</td>
<td>58.3 ± 17.4</td>
<td>58.5 ± 19.7</td>
</tr>
<tr>
<td>Low-density lipoprotein (mg/dl)</td>
<td>90.8 ± 14.5</td>
<td>89.5 ± 18.2</td>
</tr>
<tr>
<td>Triglyceride (mg/dl)</td>
<td>131.8 ± 72.9</td>
<td>144.8 ± 97.2</td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>132.6 ± 13.4</td>
<td>133 ± 10.8</td>
</tr>
<tr>
<td>Diastolic blood pressure (mmHg)</td>
<td>75.1 ± 12.7</td>
<td>74 ± 9.5</td>
</tr>
<tr>
<td>MDA-LDL (IU/L)</td>
<td>95.1 ± 25.8</td>
<td>108.9 ± 42.3</td>
</tr>
<tr>
<td>N-ε(carboxymethyl) lysine (µg/ml)</td>
<td>5.44 ± 0.55</td>
<td>5.23 ± 0.58</td>
</tr>
<tr>
<td>Lipid hydroperoxide in erythrocytes (fluorescence intensity/lipid)</td>
<td>2.12 ± 0.61</td>
<td>1.87 ± 0.64*</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SD.  
* P<0.05 vs. baseline.

MDA-LDL, malondialdehyde-modified low density lipoprotein.
erating the non-specific attacks of free radicals to lipid, the measurement of total volume of lipid hydroperoxides, not limited to any special class of lipid hydroperoxides, should be important to understand the total impact of oxidative stress.

In general, it is difficult to evaluate the oxidative stress in human precisely. While several serum markers of oxidative stress such as CML, MDA-LDL, thiobarbituric acid reactive substances, and 8-epi-prostaglandin \( \mathrm{F}_2 \alpha \) are currently available clinically, these markers do not necessarily correlate well with each other in clinical setting [36]. In fact, in the present study, while antioxidative supplement in healthy subjects resulted in a significant decrease in CML, it did not alter MDA-LDL level. The same treatment significantly decreased lipid hydroperoxides in the membrane of erythrocytes of normal subjects. On the other hand, azelnidipine decreased lipid hydroperoxides in erythrocyte membrane, but not CML or MDA-LDL. Different from other oxidative markers, lipid hydroperoxide was measured using the erythrocyte membrane in our study. Thus, lipid hydroperoxides in erythrocytes might reflect different aspects from other oxidative markers. In fact, in this study, DPPP successfully evaluated the decrease in lipid hydroperoxides in erythrocyte membrane, but not in plasma of healthy subjects after taking vitamin C and vitamin E. Further studies are necessary to elucidate the effects and clinical implication of similar treatments on each oxidative stress marker.

While DCCB is regarded to have anti-oxidative activity [7], azelnidipine is expected to have a larger anti-oxidative activity at its clinical dosage than other DCCBs [37]. In the present study, we evaluated the effectiveness of azelnidipine in terms of oxidative stress. Azelnidipine treatment had no significant effect on blood pressure, blood glucose, and lipid profile compared with amlodipine treatment. On the other hand, lipid hydroperoxide in erythrocyte membrane diminished after changing amlodipine to azelnidipine. This result clearly highlights the unique antioxidative effect of azelnidipine in diabetic patients.

Our study has certain limitations. The number of study subjects was small. In addition, because amlodipine was the most frequently prescribed DCCB in Japan, we expect that it was easier to recruit type 2 diabetes with hypertension taking amlodipine than drug naive patients as study subjects, and we compared the data before and after the change of the drug. Cross-over trial design is definitely better to compare the effect of each drug. However, eventually, to elucidate the strict clinical usefulness of azelnidipine on diabetic subjects, further studies with larger numbers of patients have to be performed to fully investigate the outcome of diabetic conditions and complications. Our data should provide useful information as a first step for elucidating the efficacy of azelnidipine.

The Japan Society of Hypertension Guidelines (JSH 2004) emphasized the importance of strict blood pressure control in patients with type 2 diabetes. The report also recommends the use of angiotensin converting enzyme (ACE) inhibitors, angiotensin II type 1 receptor blockers (ARB)s and long-acting DCCB for the patients with type 2 diabetes [38]. Although it is well known that ACE inhibitors, ARBs and the combination use of both drugs have beneficial effects on hypertension-related cardiovascular end-organ damage [39–41], it is often difficult to achieve the target blood pressure without the use of long-acting DCCB on these drugs in clinical setting. Considering the importance of oxidative stress in the pathophysiology of diabetic state and its complications, our data may provide useful information to choose suitable medication for diabetic patients among DCCB.

To the best of our knowledge, this is the first report using DPPP to evaluate lipid hydroperoxides to assess one aspect of oxidative stress in a clinical setting. Considering the restrictive condition of human studies, the method applied here may be suitable for other investigative protocols.

**Acknowledgement**

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**Duality of interest**

The authors declare that no duality of interest exists in the new findings of this study.
References

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