Effects of Ca-Antagonists on Oxidative Susceptibility of Low Density Lipoprotein (LDL)

Hiroshi Yoshida, Makoto Ayaori, Michio Suzukawa, Hiroshi Hosoi, Masato Nishiwaki, Toshitsugu Ishikawa, and Haruo Nakamura

Twelve adults (age 32-61 years) with essential hypertension were recruited from the outpatient clinics of National Defense Medical College hospital to serve as subjects in the present study. They were treated with nilvadipine, a Ca-antagonist, 4 mg b.i.d. for 4 weeks. LDL samples were isolated by ultracentrifugation at the beginning (week 0) and at the end (week 4) of the treatment regimen. The formation of conjugated dienes was measured by incubating 100 µg of LDL protein with 2 µmol CuSO₄ in 2 ml phosphate buffered saline (PBS). There were no significant differences between lipids levels, composition and anti-oxidant levels of LDL at weeks 0 and 4. The lag time of LDL oxidation was 71.1 ± 11.3 min at week 0 and 81.3 ± 13.2 min at week 4 (p < 0.05). In vitro studies of LDL oxidation, evaluated by thiobarbituric acid reactive substances (TBARS) and by agarose electrophoretic mobility, indicated that nilvadipine inhibited the oxidative modification of LDL while amloidine, used as control, did not. Nilvadipine, a lipophilic Ca-antagonist, significantly prolonged the lag time of conjugated diene formation of LDL by 12.6 % but amloidine, a hydrophilic Ca-antagonist, had no major effect on LDL oxidation. These results suggest that Ca-antagonists are effective for the prevention of atherosclerosis but the effect is dependent upon the lipophilicity of the drugs. (Hypertens Res 1995; 18: 47-53)

Key Words: Ca-antagonist, oxidative susceptibility, low density lipoprotein (LDL)

Oxidized low density lipoprotein (LDL) has been reported to play an important role in atherogenesis by direct cytotoxicity, by a chemotactic effect on monocytes, by an inhibitory effect on macrophage motility and by initiation of foam cell formation by macrophages leading to the formation of atherosclerotic plaques that take up oxidized LDL via their scavenger receptors (1-4).

Ca-antagonists and beta-blockers are effective cardiovascular drugs widely used in the treatment of angina, arrhythmias and hypertension, and their effects on the primary and secondary prevention of coronary heart diseases (CHD) have been widely discussed in the literature. Several papers have reported a positive correlation between the severity of coronary atherosclerosis and the oxidative susceptibility of LDL (5). Breugnot et al. (6) reported that Ca-antagonists prevent monocyte and endothelial cell-induced modification of LDL.

It therefore seems that not only plasma lipid levels but also the oxidative susceptibility of LDL needs to be considered in treating hypertension and associated conditions. This study investigated the possibility that Ca-antagonists afford a protective effect against LDL oxidative modification, studied in vitro and ex vivo, and thereby inhibit atherogenesis.

Methods

Subjects

Twelve nonsmoking normolipidemic or mildly hyperlipidemic adults (5 males, 7 females; age 32-61 years) with essential hypertension were recruited from the outpatient clinics of National Defense Medical College hospital. Fasting levels of total cholesterol (TC) and triglyceride (TG) in all subjects were less than 250 mg/dl and 200 mg/dl, respectively. None had diabetes mellitus, endocrine diseases, renal dysfunction or liver dysfunction, nor were taking any supplemental vitamins or medications. Mean body mass index of the 12 subjects was 23.4 ± 2.3 kg/m² and did not change significantly during the study. With the exception of hypertension, all subjects were in good physical condition and each gave informed consent to participate in the study. They were asked not to change their usual dietary habits for the duration of the study. This study was approved by the review board of the National Defense Medical College.

Experimental Design

Body height, body weight and blood pressure were recorded and fasting blood samples were drawn from each subject at the outset (week 0) and at the
end (week 4) of the study period. During the 4-week study period, each subject was treated with nilvadipine (4 mg b.i.d.), a Ca-antagonist. Venous blood samples were collected in vacutainers containing 1 mg/ml ethylenedinitrilo-tetraacetic acid (EDTA) and placed in an ice bath. Plasma was immediately separated by centrifugation, 1,500 g for 20 min at 4°C, and maintained at this temperature throughout its preparation. LDL (d = 1.019-1.063) was isolated by sequential preparative ultracentrifugation using the method of Havel et al. (7). Half of the LDL was stored in darkness under nitrogen for up to 3 days at 4°C before determination of the oxidative susceptibility of LDL, and the other half was kept at -70°C until determination of lipids, composition, and anti-oxidants in LDL.

Biochemical Analysis
Plasma or LDL TC, TG, free cholesterol (FC) and phospholipid concentrations were measured by an enzymatic method using commercially available enzymatic reagents (Kyowa Medical, Tokyo). Cholesterol ester content was calculated as 1.68 × (TC - FC). HDL-cholesterol was determined in whole plasma after precipitation of apolipoprotein B-containing lipoproteins with dextran sulfate and magnesium chloride (8). Plasma apolipoprotein A I, A II, B, C II, C III and E concentrations were measured by the immunoturbidimetry method (9-11). LDL protein concentrations were determined by the method of Lowry et al. (12).

Vitamin E (α-tocopherol) and β-carotene in LDL were measured by high performance liquid chromatography (HPLC) following methods described in earlier studies (13, 14). Nilvadipine contents in LDL were measured by HPLC. LDL lipids were extracted by a modification of the method of Folch et al. (15). The fatty acids in LDL were transmethylated and analyzed by gas chromatography (16, 17).

Determination of Oxidative Susceptibility of LDL
In Vitro Oxidation of LDL
Each LDL fraction was dialyzed extensively against phosphate buffered saline (PBS), pH 7.4 at 4°C in the dark. The LDL was oxidized with copper sulfate (CuSO4) in PBS at 37°C.

Measurement of the Formation of Conjugated Dienes in LDL
Conjugated diene formation was measured by incubating 100 µg of LDL protein dialyzed with PBS with 2 µM CuSO4 in 2 ml PBS at 37°C for 4 h. LDL oxidation kinetics were monitored by the change in 234 nm absorbance at 37°C in a spectrophotometer (Shimazu 160 A, Shimazu, Tokyo) equipped with a six-position automatic changer (5, 18). The dienes formed during LDL oxidation produce an absorption spectrum with a distinct peak at 234 nm with essentially no inter-individual variation; the initial absorbance at 234 nm was taken as the baseline and the change in absorbance was recorded every 10 min for 4 h. The absorbance curve at 234 nm was divided into 3 phases, i.e., a lag phase, a propagation phase, and a decomposition phase. The lag time of LDL oxidation was defined as the intercepts of the tangent of the slope of the absorbance curve in the propagation phase with the baseline, expressed in minutes.

The LDL, which was preincubated with Ca-antagonists (nilvadipine or amlodipine) at the indicated concentrations for 15 min at room temperature and for 60 min at 4°C, was prepared in order to determine the in vitro effects of Ca-antagonists on the oxidative susceptibility of LDL. Since nilvadipine is lipophilic, nilvadipine in an ethanol solution (final ethanol concentration 0.5%) was introduced at the beginning of the incubation period; controls contained 0.5% ethanol alone. Since amlodipine is hydrophilic, amlodipine was redissolved in distilled water; controls contained distilled water alone. The in vitro effects of Ca-antagonists on LDL oxidation with copper ions were estimated by measuring the formation of conjugated dienes and thiobarbituric acid reactive substances (TBARS), and by agarose gel electrophoretic mobility. The measurement of conjugated diene formation was accomplished as described above. In the assay of TBARS and electrophoretic mobility, 250 µg of LDL protein, which was pretreated in vitro with Ca-antagonists, was oxidized with 5 µmol copper at 37°C, and the oxidation was stopped with 0.3 µmol EDTA at 0, 1, 3, 4, 5, 6, 7 and 9 h after the beginning of LDL oxidation in the assay of electrophoretic mobility or at 0, 1, 2, 3, 4, 5, 6, 8 and 10 h in the assay of TBARS. TBARS was analyzed according to the method of Buege and Aust from the absorption at 532 nm (19). The negative surface charge of LDL (apo-protein B) was measured by agarose gel electrophoresis at 100 V for 30 min.

Statistics
Values are expressed as mean ± SD. Comparisons between means were made using Student’s t-tests for paired data or Wilcoxon rank sum tests for unpaired nonparametric data. Spearman’s correlation coefficient was calculated to test the significance of correlations between measures. Significance was established at p ≤ 0.05.

Results
Blood Pressure
As expected, administration of nilvadipine resulted in a significant (p < 0.05) decrease in mean systolic blood pressure (from 162 ± 12 mmHg at week 0 to 134 ± 14 mmHg at week 4) and mean diastolic blood pressure (from 102 ± 9 mmHg at week 0 to 86 ± 10 mmHg at week 4).

Plasma Lipids and Apoproteins
Nilvadipine had no significant effects on plasma lipid and apolipoprotein (Table 1). LDL-cholesterol values in Table 1 are estimated according the Friedewald equation (20).

LDL Composition
Nilvadipine did not significantly change values of lipids and protein in LDL, and had no major effects
Yoshida et al: LDL Oxidation and Ca-Antagonists

Table 1. Effect of Nilvadipine on Plasma Lipids and Apolipoproteins

<table>
<thead>
<tr>
<th></th>
<th>Baseline (week 0)</th>
<th>Week 4</th>
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<tbody>
<tr>
<td>Total cholesterol</td>
<td>206.4±32.6</td>
<td>193.3±27.9</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>134.9±51.4</td>
<td>131.9±48.6</td>
</tr>
<tr>
<td>HDL-cholesterol</td>
<td>51.9±13.0</td>
<td>46.3±10.4</td>
</tr>
<tr>
<td>LDL-cholesterol</td>
<td>127.5±27.2</td>
<td>120.6±23.2</td>
</tr>
<tr>
<td>Phospholipids</td>
<td>218.7±29.8</td>
<td>206.8±22.8</td>
</tr>
<tr>
<td>Apolipoprotein AI</td>
<td>142.2±23.4</td>
<td>129.8±20.1</td>
</tr>
<tr>
<td>Apolipoprotein AIi</td>
<td>38.8±8.9</td>
<td>35.0±9.5</td>
</tr>
<tr>
<td>Apolipoprotein B</td>
<td>104.9±18.6</td>
<td>102.8±24.1</td>
</tr>
<tr>
<td>Apolipoprotein CII</td>
<td>3.9±1.4</td>
<td>4.4±2.1</td>
</tr>
<tr>
<td>Apolipoprotein CIII</td>
<td>11.8±4.2</td>
<td>10.6±3.4</td>
</tr>
<tr>
<td>Apolipoprotein E</td>
<td>5.4±1.2</td>
<td>5.2±1.5</td>
</tr>
</tbody>
</table>

The data are expressed as mg/dl. Values represent mean±SD.

Table 2. Effect of Nilvadipine on LDL Composition

<table>
<thead>
<tr>
<th></th>
<th>Baseline (week 0)</th>
<th>Week 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>LDL-TC</td>
<td>130.6±44.1</td>
<td>127.7±58.4</td>
</tr>
<tr>
<td>LDL-CE</td>
<td>101.1±37.2</td>
<td>96.8±45.6</td>
</tr>
<tr>
<td>LDL-FC</td>
<td>29.5±9.5</td>
<td>30.9±13.1</td>
</tr>
<tr>
<td>LDL-TG</td>
<td>41.9±14.7</td>
<td>45.9±14.2</td>
</tr>
<tr>
<td>LDL-PL</td>
<td>87.5±32.5</td>
<td>86.9±33.8</td>
</tr>
<tr>
<td>LDL-protein</td>
<td>85.1±29.8</td>
<td>91.6±35.9</td>
</tr>
<tr>
<td>TC+TG+PL/protein</td>
<td>3.14±0.75</td>
<td>2.73±0.26</td>
</tr>
<tr>
<td>CE+TG/FC+PL+protein</td>
<td>0.72±0.17</td>
<td>0.69±0.11</td>
</tr>
</tbody>
</table>

The data are expressed as mg/dl. Values represent mean±SD. Abbreviations: TC, total cholesterol; CE, cholesterol esters; FC, free cholesterol; TG, triglycerides; PL, phospholipids.

on total lipid/protein in LDL and core/surface in LDL (Table 2). Specifically, nilvadipine did not change LDL particle size since total lipid/protein ratio and core/surface component ratio in LDL is considered to be an expression of LDL particle size (21). Fatty acid composition is considered to be an important factor in LDL oxidation. Table 3 shows that nilvadipine had no major effect on fatty acid composition in LDL.

Anti-Oxidant Levels in LDL and Lag Time of LDL Oxidation

α-Tocopherol and β-carotene levels in LDL were not changed significantly by nilvadipine, nor were the α-tocopherol/LDL protein and β-carotene/LDL protein ratios altered by nilvadipine (Table 4). However, the treatment with nilvadipine significantly (p < 0.05) increased the lag time of conjugated diene formation in LDL oxidation from 71.1 ± 11.3 min at week 0 to 81.3 ± 13.2 min at week 4.

Nilvadipine Concentrations and Oxidative Susceptibility of LDL

Nilvadipine concentrations in LDL at week 4 were 0.5 to 2.5 ng/ml and correlations between the lag time of LDL oxidation and the LDL α-tocopherol level per mg LDL protein or the LDL β-carotene level per mg LDL protein, were significant (r = 0.538, r = 0.407, respectively; p < 0.05). Accord-

Table 3. Effect of Nilvadipine on Fatty Acids in LDL

<table>
<thead>
<tr>
<th>Fatty acids</th>
<th>Baseline (week 0)</th>
<th>Week 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>SFA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C12:0</td>
<td>0.078±0.072</td>
<td>0.099±0.072</td>
</tr>
<tr>
<td>C14:0</td>
<td>0.801±0.151</td>
<td>0.836±0.236</td>
</tr>
<tr>
<td>C16:0</td>
<td>17.734±0.989</td>
<td>17.652±1.447</td>
</tr>
<tr>
<td>C18:1</td>
<td>5.700±0.531</td>
<td>5.479±0.379</td>
</tr>
<tr>
<td>C22:0</td>
<td>0.228±0.051</td>
<td>0.196±0.042</td>
</tr>
<tr>
<td>C24:0</td>
<td>0.692±0.132</td>
<td>0.174±0.185</td>
</tr>
<tr>
<td>MUFA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C14:1</td>
<td>0.182±0.024</td>
<td>0.239±0.110</td>
</tr>
<tr>
<td>C16:1</td>
<td>2.443±0.718</td>
<td>2.374±0.960</td>
</tr>
<tr>
<td>C18:1</td>
<td>17.122±2.886</td>
<td>16.076±2.666</td>
</tr>
<tr>
<td>C20:1</td>
<td>0.630±0.387</td>
<td>0.532±0.194</td>
</tr>
<tr>
<td>C24:1</td>
<td>1.154±0.548</td>
<td>1.130±0.352</td>
</tr>
<tr>
<td>PUFA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C18:2</td>
<td>38.687±4.735</td>
<td>38.992±5.082</td>
</tr>
<tr>
<td>C18:3</td>
<td>0.665±0.229</td>
<td>0.644±0.152</td>
</tr>
<tr>
<td>C20:3</td>
<td>1.267±0.317</td>
<td>1.186±0.329</td>
</tr>
<tr>
<td>C20:4</td>
<td>5.046±1.046</td>
<td>5.327±0.819</td>
</tr>
<tr>
<td>C20:5</td>
<td>1.565±0.948</td>
<td>1.538±1.030</td>
</tr>
<tr>
<td>C22:6</td>
<td>6.204±0.823</td>
<td>6.484±1.667</td>
</tr>
</tbody>
</table>

The data are expressed as % weight. Values represent mean±SD. Abbreviations: SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids.
ingly, we examined the oxidative susceptibility of LDL preincubated with nilvadipine in vitro. The lag time of conjugated diene formation was prolonged in a dose-dependent fashion by nilvadipine as compared with amlodipine (Fig. 1, Table 5). The relative electrophoretic mobility of LDL was similarly delayed by the preincubation with nilvadipine. After the beginning of oxidation with copper, the apparent increase in the negative charge of LDL was recognized at 3 h in the controls, at 4 h in the culture preincubated with 1 ng/ml of nilvadipine, and at 5 h in the culture preincubated with 10 ng/ml of nilvadipine (Fig. 2). In the assay with TBARS, nilvadipine showed a dose-dependent potential to protect against LDL oxidation (Fig. 3). However, the preincubation with amlodipine had no major effects on the relative electrophoretic mobility of LDL (Fig. 4).

### Table 4. Effect of Nilvadipine on Conjugated Diene Formation in LDL Oxidized with Copper and Levels of Antioxidants in LDL

<table>
<thead>
<tr>
<th></th>
<th>Baseline (week 0)</th>
<th>Week 4</th>
</tr>
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<tbody>
<tr>
<td>Lag time</td>
<td>71.1 ± 11.3</td>
<td>81.3 ± 13.2*</td>
</tr>
<tr>
<td>α-Tocopherol</td>
<td>429.2 ± 190.1</td>
<td>460.0 ± 230.1</td>
</tr>
<tr>
<td>β-Carotene</td>
<td>20.1 ± 11.8</td>
<td>23.1 ± 13.4</td>
</tr>
<tr>
<td>α-Tocopherol/protein</td>
<td>5.18 ± 2.32</td>
<td>5.01 ± 2.71</td>
</tr>
<tr>
<td>β-Carotene/protein</td>
<td>0.25 ± 0.15</td>
<td>0.28 ± 0.14</td>
</tr>
</tbody>
</table>

Lag times are expressed as minutes. Antioxidants are expressed as ug/dl, and the ratios of antioxidants to protein are expressed as ug/mg. Values represent mean ± SD.* p<0.05.

**Fig. 1.** Effects of Ca-antagonists incubated in vitro on oxidative susceptibility of LDL. A: Nilvadipine incubated in vitro with LDL. ●, controls; ○, incubation with 1 ng/ml nilvadipine; △, incubation with 10 ng/ml nilvadipine. B: Amlodipine incubated in vitro with LDL. ●, controls; ○, incubation with 2 ng/ml amlodipine; △, incubation with 20 ng/ml amlodipine.

**Fig. 2.** Effects of nilvadipine incubation on relative electrophoretic mobility of LDL oxidized with copper. Relative electrophoretic mobilities of LDL are recognized at 0, 1, 3, 4, 5, 6, 7, and 9 h after the beginning of copper-mediated oxidation.
Discussion

The results of the present study clearly demonstrate that nilvadipine, a Ca-antagonist, can prevent Cu²⁺-induced LDL oxidation. Although some Ca-antagonists have been previously reported to inhibit cell- and metal-ion-mediated lipid peroxidation of LDL or liposomes (6, 22), the present study is, to our knowledge, the first clinical trial to demonstrate a protective effect of a Ca-antagonist against LDL oxidation.

LDL from subjects who had been treated with nilvadipine is potentially protected against copper-induced oxidation (Table 4). This result suggests that nilvadipine is distributed in the LDL fraction. Niwa et al. (23) reported that lipoproteins and albumin are the main nilvadipine-binding proteins in human plasma. In the present study, at least part of the nilvadipine taken orally is believed to have bound to LDL. In fact, nilvadipine contents in LDL were 0.5 to 2.5 ng/ml. Although preincubation with nilvadipine in vitro could prevent LDL oxidation (Figs. 1-3), amlodipine could not (Figs. 1, 4). These drugs are in the same dihydropyridine series of Ca-antagonists, but nilvadipine is lipophilic and amlodipine is hydrophilic. Maziere et al. (24) reported that lipophilic β-blockers inhibit cell- and copper-mediated oxidation of LDL. It is possible, therefore, that the anti-oxidative effects of Ca-antagonists are dependent on their lipophilicity. However, Yue et al. (25) reported that besides lipophilicity, other mechanisms could contribute to the anti-oxidative effects of β-blockers. They reported that carvedilol, a highly lipophilic β-blocker, inhibits LDL oxidation more strongly than does propranolol (another lipophilic β-blocker) and has free radical scavenging activity, as confirmed by electron paramagnetic resonance techniques (26). It may be that nilvadipine and nifedipine, lipophilic Ca-antagonists, can scavenge free radicals. In addition to the direct protective effects of Ca-antagonists on LDL oxidation, which is probably related to the insertion of the drugs into the LDL particle, these compounds may also modulate the mechanisms by which cells are capable of altering LDL. In this way, the mechanisms by which Ca-antagonists protect LDL against copper-(and cell-)mediated oxidation are probably

![Table 5. Effects of Ca-Antagonists Incubated in vitro on Lag Time of LDL Oxidation](image)

<table>
<thead>
<tr>
<th>LDL</th>
<th>Lag time (min)</th>
</tr>
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<tbody>
<tr>
<td>Control (0.5% ethanol)</td>
<td>69.7±2.4</td>
</tr>
<tr>
<td>Nilvadipine 1 ng/ml</td>
<td>77.8±1.8</td>
</tr>
<tr>
<td>Nilvadipine 10 ng/ml</td>
<td>91.4±2.1</td>
</tr>
<tr>
<td>Control (distilled water)</td>
<td>70.3±3.2</td>
</tr>
<tr>
<td>Amlodipine 2 ng/ml</td>
<td>71.9±1.7</td>
</tr>
<tr>
<td>Amlodipine 20 ng/ml</td>
<td>71.0±3.3</td>
</tr>
</tbody>
</table>

The data of lag time are expressed as minutes. Values represent mean ± SD of triplicate measurements in each group.

a, p<0.05 vs. control (0.5% ethanol); b, p<0.01 vs. control (0.5% ethanol); c, p<0.05 vs. nilvadipine 1 ng/ml.
complex and multifocal and should be investigated further.

The anti-oxidative vitamins are very important to the study of the oxidative susceptibility of LDL. Ingestion of α-tocopherol leads to elevation of plasma and LDL α-tocopherol levels and a decreased oxidative susceptibility of LDL when tested ex vivo (27, 28). The effects of α-tocopherol, a chain-breaking antioxidant (29), on inhibition of LDL oxidation has also been demonstrated in vitro (30). Oral supplementation with α-tocopherol rendered LDL less susceptible to oxidation in vitro, as measured by resistance to copper-mediated oxidation (27). β-carotene functions as a radical trapping anti-oxidant (31) and inhibits the oxidation of LDL in vitro (32), whereas oral supplementation with β-carotene was ineffective in protecting LDL against oxidation (28, 33). In the present study, the positive correlation between the lag time of LDL oxidation and LDL α-tocopherol level per mg LDL protein or LDL β-carotene level per mg LDL protein was significant. However, there were no significant differences between week-0 and week-4 LDL antioxidant levels (Table 4), so LDL antioxidant levels did not affect the results of LDL oxidation in the present study.

According to earlier studies, free fatty acid composition affects the oxidative susceptibility of LDL. Diets rich in polyunsaturated fatty acids (PUFA) lead to high levels of PUFA in LDL particles, which should be more susceptible to lipid peroxidation (15, 34) and possibly more atherogenic (1, 2). The reports also show that diets rich in monounsaturated fatty acids (MUFA) make LDL less susceptible to oxidation and modification (15, 34). However, in the present study there were no significant differences in LDL fatty acid composition between weeks 0 and 4. Therefore, it is not likely that LDL fatty acid composition affected the LDL oxidation results in the present study.

Lipoprotein profiles rich in smaller, more dense LDL particles have been associated with increased risk of CHD (5, 35, 36). This association could be related to the increased oxidative modification of small LDL particles in vivo with increased participation of these particles in oxidative atherogenic processes. We did not examine LDL particle size directly in the present study, but we estimated LDL particle size from the ratio of core to surface components and the ratio of total lipid to protein (Table 2), and concluded that LDL particle size did not affect the LDL oxidation results.

In conclusion, these results suggest that nilvadipine and lipophilic Ca-antagonists protect human LDL against oxidation and may thereby have anti-atherogenic effects. These results also imply a high therapeutic value of Ca-antagonists in the management of hypertension since hypertensive patients are at risk to develop atherosclerosis.

Acknowledgements

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