Effects of Cacao Liquor Polyphenols on the Susceptibility of Low-density Lipoprotein to Oxidation in Hypercholesterolemic Rabbits

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The effects of cacao liquor polyphenols (CLP) on the susceptibility of low-density lipoprotein (LDL) to oxidation in hypercholesterolemic rabbits were examined. Six Japanese white rabbits which had been fed a high cholesterol diet (HCD) for 3 weeks were fed HCD containing 1% CLP for the following 10 days. The susceptibility of LDL to oxidation induced by 2,2'-azobis(4-methoxy-2,4-dimethylvaleronitrile) (V-70) was evaluated by measuring the production of conjugated dienes and thiobarbituric acid reactive substances (TBARS). The lag time was significantly prolonged from 37.7 min before intake of CLP to 42.9, 44.2 and 45.8 min after 4, 7 and 10 days of CLP intake. TBARS production after intake of CLP was also markedly reduced compared with the level before intake. There was no difference in plasma lipid concentrations comparing the levels before and after CLP intake. In conclusion, in hypercholesterolemic rabbits, orally administered CLP was absorbed and distributed to the blood, and the resistance of LDL to oxidation was thereby increased. J Atheroscler Thromb, 2000; 7: 164-168.

Key words: Cacao liquor, Polyphenols, LDL, Oxidation, Rabbits

Introduction

Cacao beans, the seeds of Theobroma cacao, are recognized as a rich source of polyphenolic substances (1-3). Recently, we reported that cacao liquor, which is prepared from fermented, dried and cracked raw bean as one of the major ingredients of chocolate and cocoa, is rich in polyphenols. We confirmed that epicatechin, catechin (4), clovamide, quercetin and their glucosides (5) are the major antioxidant components in cacao liquor. Hammerstone et al. reported that procyanidin also is a major antioxidant in chocolate and cocoa (6).

Much attention has been focused on the chemopreventive effects of polyphenolic substances. Especially, a protective role of plant polyphenols against atherosclerosis has been suggested by several epidemiological (7-9) and experimental studies (10, 11). It has been reported that defatted cacao liquor given to volunteers reduced the susceptibility of low density lipoprotein (LDL) to oxidation (12).

In the present study, we examined the effects of dietary intake of the polyphenolic fraction derived from cacao liquor on the susceptibility of LDL to oxidation in hypercholesterolemic rabbits.
Effects of Cacao Polyphenols on LDL Oxidation

Materials

Cacao liquor polyphenolic fraction (CLP)
CLP was prepared as described in a previous report (5). Cacao liquor was defatted with n-hexane and extracted with 80% v/v ethanol. The extract was concentrated and applied to a HP2MG column (Mitsubishi Kasei Co. Ltd.). In order to remove xanthine derivatives, such as theobromine and caffeine, the column was washed with 0.05% trifluoroacetic acid containing 20% v/v ethanol, then the remaining polyphenolic constituents were eluted with 80% v/v ethanol. The 80% ethanol fraction was collected, concentrated and freeze-dried. The total polyphenol concentration was determined by the Prussian blue method using epicatechin as the standard (13). CLP was prepared at concentrations of 0.1 and 1.0 mg/ml, and 0.1 ml of these solutions added to 50 mL of water. Three milliliters of 0.1 M FeSO4 in 0.1 N HCl was added to the mixture, and 3 mL of 8 mM K3Fe(CN)6 was added to the mixture after a further 20 minutes. The optical density was read after 20 minutes at 720 nm. The levels of catechins, procyanidins and xanthine derivatives were measured by the high performance liquid chromatography (HPLC) method (14). The HPLC apparatus used was a Tosoh instrument. CLP was prepared at a concentration of 0.1 mg/ml with 80 v/v % ethanol. This solution was applied to a Supelcosil™ LC-Si column (25 cm x 4.6 mm I.D., 5 mm). Elution was performed with the solvent system CH2Cl2-MeOH-HCOOH-H2O (A) 5 : 43 : 1 :1 (v/v) and (B) 41 : 7 :1 :1 (v/v) using a linear gradient from 0 to 20% A in 30 minutes, and from 20 to 100% A in 10 min, followed by isocratic elution with 100% A for 5 minutes. The concentrations of each component in this fraction are shown in Table 1.

Animals and diets
This study was approved by the Animal Committee of Meiji Seika, and animals received human care under their guidelines.
Male Japanese white rabbits, 13 weeks old, weighing 2.5-3.0 kg, were used. The animals were individually housed under controlled conditions (temperature 18 ± 1°C, humidity 65 ± 10% and a day/night cycle of 12 h). The animals were allowed free access to food and drinking water throughout the study.
The high cholesterol diet (HCD) used was obtained from Clea Co., Tokyo Japan. The nutrient composition of the HCD was as follows: protein, 17.5%; fat, 5.1%; cholesterol, 0.98%; carbohydrate, 49.5%; ash, 9.5%; dietary fiber, 11.8%; moisture, 5.9%; vitamin E, 7.9 mg/100 g; vitamin C, 31.0 mg/100 g. total polyphenols, 0.70%. Catechins and procyanidins were not detected. HCD containing 1% CLP was prepared as follows. CLP was dissolved in ethanol and sprayed evenly over the diet to obtain a final concentration of 1% w/w.

Method

Experimental protocol
The animals were fed HCD for 3 weeks. The plasma total cholesterol concentration was monitored every week. After 3 weeks, the plasma total cholesterol level was found to have increased 12- fold compared with the level before intake of HCD (1,639 ± 397 mg/dl). Instead of the HCD, HCD containing 1% CLP was fed to the animals for the following 10 days. LDL oxidation was measured before CLP intake and after 4, 7 and 10 days of CLP intake.

Characterization of plasma lipids
Plasma concentrations of total cholesterol, free cholesterol, triglycerides, free fatty acids, and phospholipids were determined by means of commercially available enzymatic method kits (Wako Pure Chemical Industries, Japan).

LDL oxidation
LDL oxidation was measured by the method of Hirano et al. (15) with slight modification. Samples of peripheral blood were collected from the ear vein of each animal into tubes containing EDTA (final concentration, 1 mg/ml). Plasma was prepared immediately by centrifugation and used for isolation of the LDL fraction. Lipoproteins were isolated from plasma by sequential ultracentrifugation at selected densities (d<1.006 g/ml for very low density lipoprotein (VLDL), d=1.006-1.020 for intermediate density lipoprotein (IDL), d=1.021-1.060 for LDL and d>1.061 for high density lipoprotein (HDL)) according to the method of Havel et al. (16). The protein concentration in the LDL preparations was determined by the bichinchoninic acid method using bovine albumin as the standard. The isolated rabbit LDL fraction (35 μg protein/ml) and 200 μM V-70 (2-2′-azobis(4-methoxy)-2, 4-dimethylvaleronitrile) as a radical initiator were incubated at 37°C. The kinetics of LDL oxidation was determined by monitoring the change in absorbance at 234 nm due to conjugated diene formation using a Beckman Model DU 650 spectrophotometer. The lag time, which is the phase during

Table 1. Composition of polyphenol fraction derived from cacao liquor

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
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<tbody>
<tr>
<td>Total polyphenols</td>
<td>49.5 w/w %</td>
</tr>
<tr>
<td>Catechin</td>
<td>0.48</td>
</tr>
<tr>
<td>Epicatechin</td>
<td>1.83</td>
</tr>
<tr>
<td>Procyanidin B2</td>
<td>1.69</td>
</tr>
<tr>
<td>Procyanidin C1</td>
<td>2.37</td>
</tr>
<tr>
<td>Cinnamantannin A1</td>
<td>2.01</td>
</tr>
<tr>
<td>Caffeine</td>
<td>N.D.</td>
</tr>
<tr>
<td>Theobromine</td>
<td>N.D.</td>
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</tbody>
</table>

1Total polyphenol concentration was measured by Prussian blue method using epicatechin as the standard.
which the diene levels did not increase or only increased very slowly, and the propagation rate were calculated by the method of Esterbauer et al. (17).

At the same time, LDL and V-70 were incubated at 37°C for 2 hr, and TBARS formation was measured by the method of Ohkawa et al. (18). The reaction mixture contained 0.1 ml of LDL solution, 0.2 ml of 8.1% sodium dodecyl sulfate (SDS), 1.5 ml of 20% acetic acid buffer (pH 3.5). This mixture was heated at 95°C for 60 minutes. After cooling with tap water, 5.0 ml of n-butanol were added and the mixture was shaken vigorously. After centrifugation at 3,000 rpm for 10 minutes, the absorbance of the organic layer was measured at 532 nm.

Statistical analysis
Results were expressed as mean±SD. All analyses were performed using SPSS Statistical Software. The significance of differences in mean values was determined by ANOVA and multiple range comparisons or a paired t-test. Values of p < 0.05 were considered significant.

Results

LDL oxidation
Conjugated diene production
Fig. 1 shows the typical pattern of conjugated diene production induced upon incubation of V-70 with LDL. Compared with the findings before intake of CLP, the lag time, which is the phase during which the diene levels did not increase or only increased very slowly, was prolonged 1.15-fold at 4 days, 1.24-fold at 7 days and 1.32-fold at 10 days after the start of the CLP-containing diet. The propagation rate in the phase during which the diene levels very rapidly increased to a maximum value was also reduced after CLP intake compared with the findings before intake. The mean and standard deviation of the lag time and the propagation rate at each point are shown in Table 2. The lag time was significantly prolonged from 37.8±3.2 min before intake to 42.9±4.1 min, 44.2±4.3 min and 45.8±4.6 min at 4, 7 and 10 days after the start of feeding the CLP-containing diet. The propagation rate was significantly lower after 7 and 10 days of CLP intake compared with the rate before CLP intake.

TBARS production
Fig. 2 shows the TBARS production induced upon incubation of V-70 with the LDL fraction at 37°C for 2 hr. Daily intake of CLP resulted in a significant decrease in TBARS production in the LDL fraction. There was a significant negative correlation between the lag time and the TBARS concentration (r = -0.838).

Plasma lipids
The concentrations of plasma lipids are shown in Table 3. There were no significant differences in total cholesterol, free cholesterol, triglyceride, free fatty acid or phospholipid levels in plasma comparing the levels before and after CLP intake.

Table 2. Effect of CLP supplementation on LDL oxidizability

<table>
<thead>
<tr>
<th></th>
<th>Lag time (min)</th>
<th>Propagation rate (nmol dienes/min/mg protein)</th>
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<tbody>
<tr>
<td>before intake</td>
<td>37.8±3.2</td>
<td>12.5±2.0</td>
</tr>
<tr>
<td>after 4 days intake</td>
<td>42.9±4.1*</td>
<td>9.4±4.2</td>
</tr>
<tr>
<td>after 7 days intake</td>
<td>44.2±4.3*</td>
<td>9.5±2.8*</td>
</tr>
<tr>
<td>after 10 days intake</td>
<td>45.8±4.6**</td>
<td>6.1±4.2**</td>
</tr>
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*Values are means±SD (n=6). Significant difference from control; *: p<0.05, **: p<0.01. LDL oxidation was induced by 200 μM V-70 and conjugated diene production was monitored at a wave length of 234 nm.

Fig. 1. Typical kinetics of conjugated diene production induced by V-70 before CLP intake (day 0) and after 4, 7 and 10 days of CLP intake.
A mixture consisting of the LDL fraction (35 μg protein/ml) from hypercholesterolemic rabbits and 200 μM V-70 was incubated at 37°C. Conjugated diene production in the mixture was monitored by measuring the absorbance at 234 nm.

Fig. 2. TBARS production induced by V-70 before CLP intake and after 4, 7 and 10 days of CLP intake.
Values are means±SD (n=6). Significant difference compared with the findings before intake; *: p<0.05, **: p<0.01.
Discussion

Atherosclerosis is promoted by genetic and environmental factors that contribute to elevated levels of LDL, the major carrier of cholesterol in human blood. Recent evidence suggests that LDL oxidation plays an important role in the pathogenesis of atherosclerosis (19–21). Basic research has shown that oxidized LDL is more cytotoxic to arterial cells, and may cause endothelial cell damage and initiate atherogenesis (22, 23).

Epidemiological studies on the relationship between dietary antioxidant intake, plasma concentrations of antioxidants, and cardiovascular diseases have shown that these factors are associated with an increase in the incidence of atherosclerosis (24, 25). A study of 805 Dutch subjects revealed that flavonoid intake was inversely associated with morbidity and mortality from coronary heart disease (7, 8). A French paradox is that low mortality from coronary heart disease was found to be negatively correlated with the consumption of saturated fat. According to recent research findings, there is an association between this low risk of occurrence of CHD and red wine consumption. One of the components of red wine effective in preventing CHD was suggested to be polyphenols such as procyanidins and catechins (26, 27).

In the present study, we investigated the effects of daily intake of polyphenols derived from cacao liquor on indicators of atherosclerosis in hypercholesterolemic rabbits. The susceptibility of LDL to oxidation was lower after 4 days of CLP intake as compared with the findings before intake, and this was still evident after 7 or 10 days of CLP intake (Table 2, Fig. 1). The propagation rate was also significantly lower 7 and 10 days after the start of feeding the CLP-containing diet compared with the rate before intake. TBARS production in the presence of LDL is shown in Fig. 2; CLP intake increased the resistance of LDL to oxidation in hypercholesterolemic rabbits. There were no significant changes in plasma lipid levels throughout the experimental period, suggesting that CLP had a direct effect on the LDL particle as an antioxidant. In order to suppress the oxidation of LDL, the orally consumed polyphenolic substances must be absorbed from the gastrointestinal tract and distributed to the blood. Epicatechin, one of the major antioxidant components of CLP, is reported to be absorbed, and its metabolites such as methylated, sulfate-conjugated and glucuronide-conjugated forms have been detected. The concentration of non-conjugated EC in plasma was found to be very low (28, 29). In our recent report, the level of epicatechin related compounds in plasma increased and the plasma antioxidative activity was also increased after cocoa powder ingestion in rats (30).

In order to suppress the oxidation of LDL, the orally consumed polyphenolic substances must be absorbed from the gastrointestinal tract and distributed to the blood. Effects of Cacao Polyphenols on LDL Oxidation

<table>
<thead>
<tr>
<th>Table 3. Plasma lipid concentrations</th>
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<tr>
<td></td>
</tr>
<tr>
<td>before intake</td>
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<tr>
<td>after 4 days intake</td>
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<tr>
<td>after 7 days intake</td>
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<tr>
<td>after 10 days intake</td>
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1Values are means ± SD (n = 6).

In conclusion, as demonstrated here in hypercholesterolemic rabbits, upon daily oral intake of polyphenols derived from cacao liquor, the antioxidants are absorbed...
and distributed to the blood, and the resistance of LDL to oxidation is increased. Further studies are required to elucidate whether cacao liquor polyphenols are effective in preventing atherosclerosis and cardiovascular disease.

References

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