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Black-Tea Polyphenols Decrease Micellar Solubility of Cholesterol in Vitro and Intestinal Absorption of Cholesterol in Rats

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Administration of black-tea polyphenols (BTP) simultaneously reduced lymphatic recovery of both ^{3}H-cholesterol and ^{14}C-trioleoylglycerol in rats that were cannulated in the thoracic duct. BTP decreased the in vitro micellar solubility of cholesterol in a dose-dependent manner. When purified theaflavins, which are components of BTP, were used, theaflavin-monogallates (TFMGs), theaflavin-3-gallate (TF3G), and theaflavin-3′-gallate (TF3′G) were effective in eliminating cholesterol from bile salt micelles in vitro. Theaflavin (TF) and theaflavin-3,3′-digallate (TFDG) had no effect on the micellar solubility of cholesterol. The concentration of bile acid in the micelles was not influenced by the addition of any BTPs or theaflavins. These results suggest that the reduction of micellar cholesterol by BTP could be important to reducing cholesterol absorption.

KEYWORDS: Black-tea polyphenols; theaflavins; cholesterol absorption; rats

INTRODUCTION

Black-tea is one of the most popular beverages consumed worldwide. Black-tea is produced from the leaves of *Camellia sinensis*, and is fully fermented and oxidized. Catechins are the major polyphenols in green-tea. During the fermentation and oxidization process, catechins are oxidized to theaflavins and thearubigins (*1*). Theaflavins are categorized into the following forms: theaflavin (TF), theaflavin-3-gallate (TF3G), theaflavin-3′-gallate (TF3′G), and theaflavin-3, 3′-digallate (TFDG). Although green-tea catechins have been shown to have various health benefits, which include hypocholesterolemic (*2*−*8*), hypotriacylglycerolemic (*8*, *9*), antiatherogenic (*10*), antiobesity (*11*−*13*), antioxidative (*14*, *15*), and anticarcinogenic (*16*, *17*) activities, those of black-tea polyphenols (BTP) have rarely been studied (*18*−*24*). We previously reported that green-tea catechins with a galloyl moiety were effective in eliminating cholesterol from bile salt micelles in vitro and reduced lymphatic recovery of cholesterol in rats that were cannulated in the thoracic duct (*2*, *3*). This may be a cause of increased fecal excretion of cholesterol in experimental animals (*5*−*7*) and hypocholesterolemic activity in experimental animals and humans (*4*−*8*). Although it is expected that BTP are able to inhibit cholesterol absorption, studies on hypocholesterolemic activity were scarce (*21*−*24*). Vermeer et al. reported that theaflavins, especially TF3G, reduced the incorporation of cholesterol into mixed micelles in vitro (*23*). Yang et al. showed that black-tea extract reduced plasma total cholesterol concentration in rats (*21*). Davies et al. (*22*) and Maron et al. (*24*) showed the cholesterol-lowering effect of black-tea and theaflavin-enriched green-tea extract in human studies. However, in these studies, it was not evident which BTP was responsible for the physiological functions. In this study, the effect of BTP on lymphatic absorption and micellar solubility of cholesterol was examined in rats and in vitro, respectively. Since the increase of plasma cholesterol concentration is an independent risk factor for atherosclerosis (*25*), using food components that can decrease plasma cholesterol levels may be an effective strategy in preventing atherosclerosis via hypocholesterolemic activities.

MATERIALS AND METHODS

Preparation of BTPs and Isolation of Theaflavins from Black-Tea Leaves. Three kinds of dried black-tea leaves (Nuwara-Eliya, Dimbula, and Hunan) were purchased. The degree of fermentation and oxidation was different among the three leaves. The degree of fermentation and oxidation was lowest in Nuwara-Eliya and highest in Hunan. Each of them (200 g) was immersed in 2 L of 60% ethanol at room temperature for 1 h. These solutions were applied onto 0.6 L of hydrophilic vinylpolymer (Toyopearl HW-40EC; Tosoh, Tokyo, Japan), respectively, and then these samples were eluted using five beds of 60% ethanol. The third to fifth bed fractions were evaporated and lyophilized. Caffeine was largely eluted in the first to second bed fractions. The extraction yields were Nuwara-Eliya, 4.8 g; Dimbula, 2.7 g; and Hunan, 8.2 g, respectively from 200 g of dried black-tea leaves. Catechins, theaflavins, and caffeine contents in these BTPs were analyzed by high-performance liquid chromatography (HPLC) with UV detection at 280 nm (*19*). An Xbridge Shield RP18 column (4.6 mm × 150 mm, 3.5 μm particle size, Waters, Milford, MA) maintained at 40 °C was used. A ternary-solvent system (solvent A, ultrapure water; solvent B, acetonitrile; solvent C, 1.0% phosphoric acid in pure water) was used, and the flow rate was maintained at 1.0 mL/min. After sample injection, the phase composition was changed to obtain the following gradients: 85%/5%/10% at 0 min, 60%/30%/10% at 30 min, 50%/40%/10% at 40 min, 30%/60%/10% at 50 min and 85%/5%/10% at 60 min of solvents A/B/C, respectively. The composition of catechins and theaflavins in the BTP fraction was established by using standard curves of pure catechins and theaflavins purchased from Wako (Wako Pure Chemicals,

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Osaka, Japan) and Nagara Science (Gifu, Japan), respectively. Total polyphenols were measured using the Folin−Denis method (*26*). The composition of BTPs is shown in **Table 1**. To obtain sufficient amounts of pure TF, TF3G, TF3′G, and TFDG for in an vitro study, a part of the BTP fraction was then subjected to HPLC using a Wakosil- II 5C18HG column (20 mm × 250 mm: Wako Pure Chemicals) with UV detection at 280 nm. The solvent comprised 23% acetonitrile, 3% ethylacetate, 0.5% acetic acid, and 73.5% ultrapure water, and the flow rate was maintained at 10 mL/min. The chemical structures of the four purified theaflavins were verified using ^{1}H NMR, ^{13}C NMR and fast atom bombardment mass spectrometry (FABMS). The structures were the same as those reported previously (*27*). The purity of all of these theaflavins was greater than 96%.

Lymphatic Recoveries of ^{3}H-Cholesterol and ^{14}C-Trioleoylglycerol in Rats Cannulated in the Thoracic Duct. Before cannulation, 8-week-old male Sprague−Dawley (SD) rats (CLEA Japan, Inc., Tokyo, Japan) were fed a commercial chow for 1 week. The left thoracic lymphatic duct cephalad up to the cisterna chyli was cannulated after the rats were anesthetized with Nembutal (Dainippon Sumitomo Pharma Co., Ltd., Osaka, Japan), as described previously (*9*). A second indwelling catheter was placed in the stomach for administering the test emulsion. After surgery, the animals were placed in restraining cages, and a solution containing 139 mmol/L glucose and 85 mmol/L NaCl was intragastrically administered continuously at a rate of 3.4 mL/h until the end of the experiment. The same solution was provided as drinking water. The following morning, animals with a constant lymph-flow rate were administered 3 mL of a test emulsion containing ^{3}H-cholesterol and ^{14}C-trioleoylglycerol (PerkinElmer Inc., Wellesley, MA) with or without BTP (Hunan). The test emulsion (3 mL) contained 200 mg of sodium taurocholate (Nacalai Tesque, Kyoto, Japan), 50 mg of fatty acid-free BSA fraction V (Bayer Corp, IL), 5.56 mg of carboxymethylcellulose, 20 mg of cholesterol (Sigma, St. Louis, MO), 200 mg of trioleoylglycerol (Sigma), 185 kBq of ^{3}H-cholesterol and 37 kBq of ^{14}C-trioleoylglycerol. The mixture was then emulsified by sonication. BTP was added in the emulsion at a concentration of 50 mg/3 mL. Lymph was fractionally collected in ice-chilled tubes containing ethylenediaminetetraacetic acid (EDTA) at 0−3, 3−6, 6−9 and 9−24 h after a test emulsion was administered, respectively, and the volumes were measured. Each fraction of lymph (0.5 mL) was mixed with 10 mL of liquid scintillation cocktail (ACS II, GE Healthcare UK Ltd., Buckinghamshire, England), and radioactivity was measured using a liquid scintillation counter (LSC-5100, Aloka Co Ltd., Tokyo, Japan). Lymphatic recovery (%) was calculated as follows: The amount of the radioactivity recovered from lymph was divided by that in the emulsion administered to the stomach, and it was converted to a percentage. At the end of the study, rats were killed by injecting an excess of Nembutal (Dainippon Sumitomo Pharma Co., Ltd.). All the rats used in the study were cared for according to the guidelines for animal experiments of the Faculty of Agriculture, Graduate School Tohoku University and Law 105 and Notification 6 of the government of Japan.

Table 1. Composition of BTPs

	g/100 g dry weight		
	Nuwara-Eliya[a]	Dimbula[a]	Hunan[a,b]
TF[c]	1.5	0.5	4.8
TF3G[c]	1.2	3.2	3.4
TF3′G[c]	1.6	2.4	8.1
TFDG[c]	2.6	5.3	8.8
total teaflavins	6.9	12.4	25.1
EGCG[c]	27.6	12.0	1.4
GCG[c]	3.4	3.8	2.0
ECG[c]	13.1	11.0	9.0
CG[c]	2.2	3.1	1.2
EGC[c]	5.4	0.6	0.2
GC[c]	1.0	0.4	0.0
EC[c]	4.0	1.1	0.5
(+)C[c]	1.1	0.4	0.2
total catechins	57.8	32.4	14.5
caffeine	nd[d]	nd	0.1
total polyphenols	94.9	94.7	84.1

[a] Used in an in vitro study. [b] Used in a rat study. [c] TF: theaflavin. TF3G: theaflavin-3-gallate. TF3′G: theaflavin-3′-gallate. TFDG: theaflavin-3,3′-digallate. EGCG: (−)-epigallocatechin gallate. GCG: (−)-gallocatechin gallate. ECG: (−)-epicatechin gallate. CG: (−)-catechin gallate. EGC: (−)-epigallocatechin. GC: (−)-gallocatechin. EC: (−)-epicatechin and (+)C: (+)-catechin. [d] Not detected.

Micellar Solubility of Cholesterol in Vitro. The effects of three kinds of BTP and pure theaflavins on micellar solubility of cholesterol were examined according to our previous study (*3*). A bile salt micellar solution containing 6.6 mmol/L sodium taurocholate, 0.6 mmol/L egg yolk phosphatidylcholine (Sigma), 0.5 mmol/L cholesterol, 132 mmol/L NaCl, and 15 mmol/L sodium phosphate at pH 6.8 was prepared by sonication and kept at 37 °C for more than 24 h. Various amounts of BTPs in deionized water (100 μL) kept at 37 °C were added to the 3 mL micellar solution. The amounts of BTPs added were adjusted to 50, 100, and 200 μg/mL micelles. Various amounts of pure theaflavins in 20% ethanol solution (100 μL) kept at 37 °C were added to the 3 mL micellar solution. The amounts of pure theaflavins added were adjusted to 0.25 and 0.5 mmol/L micelles. The molar ratios of each theaflavin to cholesterol were 0.5 and 1. The mixture was incubated for 1 h at 37 °C. The solution was passed through a 0.2 μm syringe filter (25 mm; GDD/X; Whatman Inc., Clifton, NJ), and the filtrate obtained was subjected to cholesterol

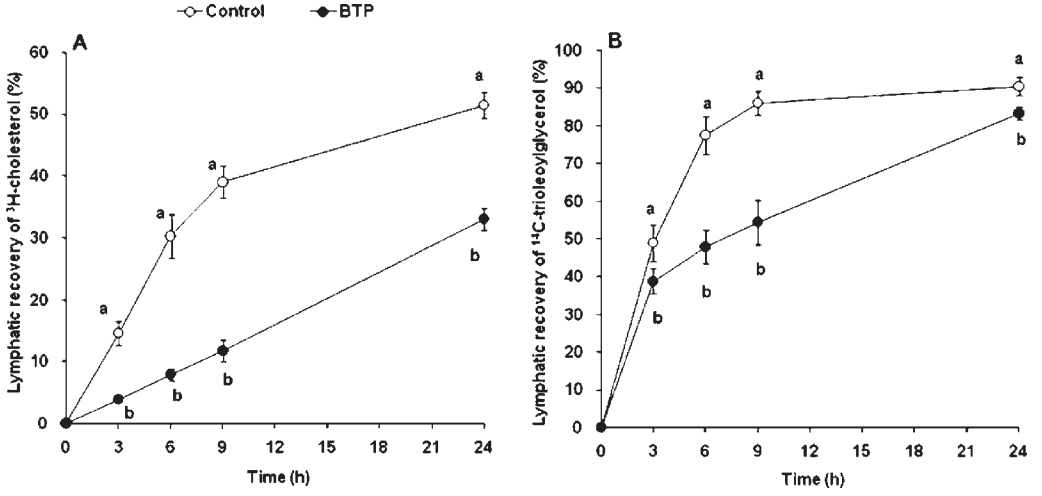

Figure 1. Effect of black-tea polyphenols (BTP) on lymphatic recovery of ^{3}H-cholesterol (**A**) and ^{14}C-trioleoylglycerol (**B**) in rats intragastrically administered a fat emulsion: ○, control group; ●, BTP group. BTP (Hunan) was intragastrically administered at 50 mg/rat together with a fat emulsion at 3 mL/rat. Lymphatic recovery (%) was calculated as follows: The amount of radioactivity recovered from lymph was divided by that in the emulsion administered to the stomach, and it was converted to a percentage. Data are means ± SE of 6 rats. Means not sharing a common letter at a time point significantly differ at $P < 0.05$.

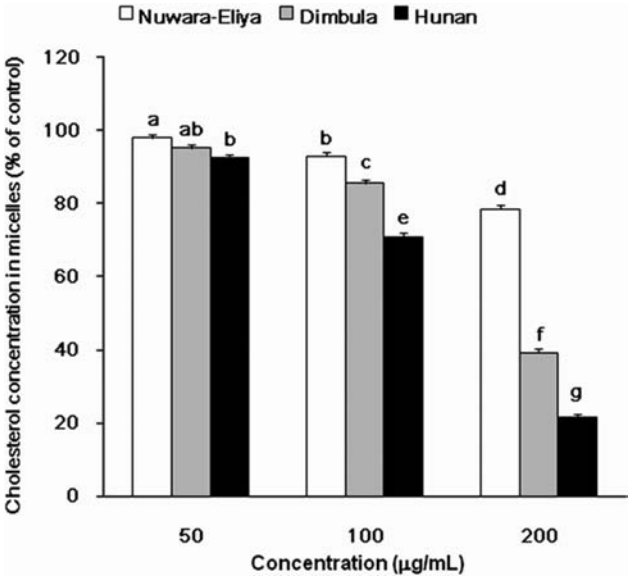

Figure 2. Effect of three kinds of black-tea polyphenols (BTP) on micellar solubility of cholesterol in vitro. Various amounts of BTPs in deionized water (100 μL) kept at 37 °C were added to the 3 mL micellar solution. The micellar cholesterol concentration at 0 μg/mL of BTPs is estimated to be 100% (control). BTPs were added at a concentration of 50, 100, and 200 μg/mL. Data are means ± SE of triplicate experiments. Means not sharing a common letter at a concentration or a BTP significantly differ, $P < 0.05$. The letter "a" indicates no significant difference from the control. Two-way ANOVA: effect of type of BTP, $P < 0.0001$; effect of BTP concentration, $P < 0.0001$; interaction between type of BTP and BTP concentration, $P < 0.0001$.

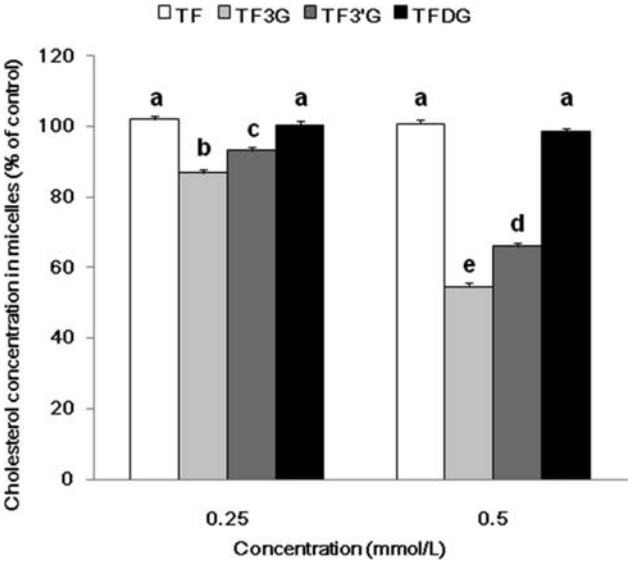

Figure 3. Effect of purified theaflavins on micellar solubility of cholesterol in vitro. Various amounts of pure theaflavins in 20% ethanol solution (100 μL) kept at 37 °C were added to the 3 mL micellar solution. The micellar cholesterol concentration at 0 mmol/L of pure theaflavins is estimated to be 100% (control). Theaflavin (TF), theaflavin-3-gallate (TF3G), theaflavin-3′-gallate (TF3′G), and theaflavin-3,3′-digallate (TFDG) were added at a concentration of 0.25 and 0.5 mmol/L. Data are means ± SE of triplicate experiments. Means not sharing a common letter at a concentration or a theaflavins significantly differ, $P < 0.05$. The letter "a" indicates no significant difference from the control. Two-way ANOVA: effect of type of theaflavins, $P < 0.0001$; effect of theaflavins concentration, $P < 0.0001$; interaction between type of theaflavins and theaflavin concentration, $P < 0.0001$.

analysis by gas chromatography using an SPB-1 column (Supelco, PA). The concentration of bile acids in the micelles was measured enzymatically using hydroxysteroid dehydrogenase (Sigma) (*28*).

Statistical Analysis. Data are expressed as means ± SE. Statistical analysis of data was performed by the Student's *t* test or two-way ANOVA followed by the Tukey−Kramer test to evaluate significant differences between a pair of means. Differences were considered significant at $P < 0.05$.

RESULTS

Lymphatic Recoveries of Cholesterol and Trioleoylglycerol in Rats Cannulated in the Thoracic Duct. Lymph flow rates were linear, and there was no difference in the rates between the control and the BTP groups (145 ± 14 and 180 ± 11 mL/24 h, respectively). Lymphatic recoveries of the radioactive ^{3}H-cholesterol and ^{14}C-trioleoylglycerol at 3, 6, 9, and 24 h after BTP administration were significantly lower in the BTP group than in the control group, respectively (**Figure 1A** and **B**).

Micellar Solubility of Cholesterol in Vitro. All of the BTPs decreased the micellar solubility of cholesterol in a dose-dependent manner (**Figure 2**). Significant differences in the effect of BTPs on micellar solubility of cholesterol were observed in the addition at concentrations of 100 and 200 μg/mL micelles. The concentration of micellar cholesterol was lowest in the addition of Hunan BTP and highest for Nuwara-Eliya BTP. When purified theaflavins, which are components of BTP, were used, we observed that TFMGs, TF3G, and TF3′G, were effective in eliminating cholesterol from bile salt micelles in vitro when added at concentrations of 0.25 and 0.5 mmol/L micelles (**Figure 3**). TF and TFDG had no effect on micellar solubility of cholesterol at 0.25 and 0.5 mmol/L. The concentration of bile acid in micelles was not influenced by the addition of any BTP or theaflavins (data not shown).

DISCUSSION

This is the first report of BTP-mediated inhibition of cholesterol absorption in rats. Since lymphatic recovery of cholesterol was reduced by the administration of BTP (**Figure 1A**), a major cause of BTP-mediated hypocholesterolemic activity in experimental animal and humans (*21*, *22*, *24*) may be the inhibition of cholesterol absorption in the intestine. BTP dose-dependently decreased the micellar solubility of cholesterol in vitro (**Figure 2**), as in the case of green-tea catechins (*2*, *3*). Addition of BTP to a bile salt micellar solution did not affect bile acid concentration in the micelles (data not shown) in the same way as catechins (*2*, *3*). This shows that BTPs do not eliminate bile acid from the bile salt micellar solution. These observations strongly suggest that transport reduction of cholesterol to the lymphatic fluid is caused by the elimination of cholesterol from bile salt micelles.

In the present study, the differential effect on the micellar solubility of cholesterol was observed according to the degree of fermentation and oxidation among the BTPs (**Figure 2**). Furthermore, when pure theaflavins were added at 0.25 and 0.5 mmol/L micelles, TF3G and TF3′G, which have a galloyl moiety, significantly decreased the micellar solubility of cholesterol in a dose-dependent manner. However, TF, which does not have the galloyl moiety, and TFDG, which has two galloyl moieties, did not reduce the micellar solubility of cholesterol (**Figure 3**). Vermeer et al. (*23*) reported that theaflavins, especially TF3G, reduced the incorporation of cholesterol into mixed micelles and existed in the pellet of the mixed micelles after centrifugation in an in vitro study. These observations suggest that TFMGs are effective components in reducing the micellar solubility of cholesterol. These findings can also explain the differential effects on the micellar solubility of cholesterol among the three kinds of BTP

used in this study (**Figure 2**); the higher the amount of TFMGs, TF3G plus TF3′G, in BTPs, the lower the micellar solubility of cholesterol (**Table 1** and **Figure 2**). However, the mode of association between TFMGs and cholesterol is not understood. When BTPs or TFMGs were added in the bile salt micellar solution (**Figures 2** and **3**), the solution immediately turned turbid and precipitates were observed. Therefore, we speculated that TFMGs can hydrophobically interact with cholesterol. However, this cannot be explained solely by the differential values of the calculated octanol/water partition coefficient (log *P*, TF, 2.050; TF3G, 4.051; TF3′G, 4.228; and TFDG, 6.228), which is a representative index of hydrophobicity, obtained from SciFinder (American Chemical Society, ACS). Therefore, more detailed stereochemical studies are necessary to determine how TFMGs associates with cholesterol.

BTPs contained about 50% of unknown polyphenols besides catechins and theaflavins, as shown in **Table 1**. There is a possibility that these unknown polyphenols in BTPs can also be effective in decreasing the micellar solubility of cholesterol in a similar manner of TFMGs.

We have previously reported that green-tea catechins with a galloyl moiety reduced the micellar solubility of cholesterol in vitro and inhibited lymphatic absorption of radiolabeled cholesterol in rats (*2*, *3*). Although Nuwara-Eliya BTP contained the highest amounts of catechins with a galloyl moiety among the three kinds of BTP (**Table 1**), its influence on the micellar solubility of cholesterol was weakest (**Figure 2**). The results suggest that TFMGs are relatively more effective in reducing intestinal absorption of cholesterol than catechins with a galloyl moiety. Comparative studies are necessary to reveal differences in the inhibitory effect on cholesterol absorption.

In the present study, the lymphatic recovery of trioleoylglycerol was also reduced by the administration of BTP (**Figure 1B**). This result confirms our previously study (*19*) and presents the possibility that BTP can simultaneously inhibit both cholesterol and triacylglycerol absorption.

We previously reported that BTP suppressed triacylglycerol absorption in rats (*19*). In that study, we administered 50 mg of BTP to rats. In this study, we administered the same amount of BTP to rats to examine whether BTP can simultaneously inhibit cholesterol and triacylglycerol absorption. Typically, a cup of black-tea comprises approximately 50−100 mg of BTP. Therefore, the amount of BTP administered to rats was nearly equivalent to that present in a half to full cup of black-tea. In our study, we administered relatively high amounts of black-tea components in order to obtain a conspicuous difference. We previously showed that green-tea catechins suppressed cholesterol absorption via the decrease of micellar solubility of cholesterol as in the case of BTP (*2*, *3*). In those studies, we administered 100 mg of green-tea catechins to rats. Kajimoto et al. showed that the catechins reduced serum cholesterol levels in mild and borderline hypercholesterolemia patients at 394 mg/capita/day in humans (*4*). Since the mechanism underlying the inhibition of cholesterol absorption by BTP is similar to that mediated by green-tea catechins, we expect that, as in the case of green tea catechins, a reasonable amount of BTP can reduce serum cholesterol in humans. In future studies, we need to examine the physiological functions of BTP and their side effects in humans.

Studies on the safety of BTP are scarce. Maron et al. showed that administration of a capsule containing a theaflavin-enriched green-tea extract (75 mg of theaflavins, 150 mg of green-tea catechins, and 150 mg of other tea polyphenols) to humans over a 12-week period did not elicit any serious adverse effects (*24*). Because information on the absorption and metabolism of BTP and theaflavins is very limited (*29*), more studies on the safety, absorption, and metabolism of these compounds should be performed in the future.

In conclusion, our study reveals the possibility that BTP may be effective in lowering plasma cholesterol concentration—an independent risk factor for atherosclerosis (*25*)—by inhibiting cholesterol absorption in the intestine. At least, a part of the inhibition is ascribed to the decrease in the micellar solubility of cholesterol. BTP have a cholesterol-lowering activity in humans (*22*, *24*), a suppressing activity on postprandial hypertriacylglycerolemia in rats (*19*), and preventative activity on low-density lipoprotein (LDL) oxidation in vitro (*22*). Therefore, BTP may be used as food components to prevent coronary heart disease.

ABBREVIATIONS USED

BTP, black-tea polyphenols; TF, theaflavin; TF3G, theaflavin-3-gallate; TF3′G, theaflavin-3′-gallate; TFDG, theaflavin-3,3′-digallate; TFMGs, theaflavin-monogallates ; HPLC, high-performance liquid chromatography; FABMS, fast atom bombardment mass spectrometry; BSA, bovine serum albumin; EDTA, ethylenediaminetetraacetic acid.

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