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Black-Tea Polyphenols Suppress Postprandial Hypertriacylglycerolemia by Suppressing Lymphatic Transport of Dietary Fat in Rats

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Administration of black-tea polyphenols (BTP) at 100 and 200 mg/kg of body weight in rats suppressed postprandial hypertriacylglycerolemia in a dose-dependent manner. Administration of BTP also suppressed lymphatic recovery of ^{14}C-trioleoylglycerol in rats that were cannulated in the thoracic duct. BTP dose-dependently inhibited the activity of pancreatic lipase in vitro with an IC_{50} of 0.254 mg/mL. When purified theaflavins, which are components of BTP, were used, theaflavins with galloyl moieties, but not those without galloyl moiety, inhibited the activity of pancreatic lipase. Theaflavin-3,3′-digallate (TFDG) was more effective in inhibiting the activity of pancreatic lipase than epigallocatechin gallate (EGCG), epicatechin gallate (ECG), and a mixture of EGCG and ECG. BTP and TFDG had a similar effect in inhibiting the activity of pancreatic lipase when the total polyphenol amount was adjusted to the same. BTP had no effect on micellar solubility of hydrolysis products of triacylglycerol. These results suggest that BTP suppressed postprandial hypertriacylglycerolemia by reducing triacylglycerol absorption via the inhibition of pancreatic lipase activity.

KEYWORDS: Black-tea polyphenols; theaflavins; postprandial hypertriacylglycerolemia; pancreatic lipase; rats

INTRODUCTION

Tea drinks are the most popular beverages consumed worldwide and are categorized as green, oolong, and black teas, which are nonfermented, partially fermented, and completely fermented/oxidized, respectively (*1*). Catechins are the major polyphenols in green tea. During the fermentation/oxidization process, catechins are oxidized to theaflavins and thearubigins (*2*). Theaflavins are categorized into the following forms: theaflavin (TF), theaflavin-3-gallate (TF3G), theaflavin-3′-gallate (TF3′G), and theaflavin-3,3′-digallate (TFDG). Green-tea catechins have been shown to have various physiological functions: they are known to possess hypocholesterolemic (*3*−*9*), hypotriacylglycerolemic (*9*, *10*), antiatherogenic (*11*), antiobesity (*12*−*14*), antioxidative (*15*, *16*), and anticarcinogenic (*17*, *18*) activities. There are very few studies that have focused on the physiological effects of oolong- and black-tea polyphenols (BTP), whereas the physiological effects of green-tea catechins have been considerably reported. Recently, researchers have focused on the physiological functions of polyphenols (*19*−*23*). Nakai et al. showed that various tea polyphenols, including theaflavins, have an inhibitory effect on pancreatic lipase activity in vitro (*19*). Although the results suggest the possibility that oolong-tea polyphenols and BTP have an ability to delay or inhibit fat absorption in the intestine and suppress postprandial hypertriacylglycerolemia, this effect has been investigated in oolong-tea polyphenols only (*23*). We previously reported that green-tea catechins with a galloyl moiety suppressed postprandial hypertriacylglycerolemia by delaying lymphatic transport of dietary fat in rats (*10*) and suppressed postprandial hypertriacylglycerolemia in humans (*24*, *25*). Because postprandial hypertriacylglycerolemia is a risk factor for coronary heart disease (*26*), its suppression by food consumption may be an effective strategy to prevent the development of coronary heart disease. In this study, the effect of BTP on postprandial hypertriacylglycerolemia was examined in rats, and the underlying mechanism of the action of BTP was elucidated both in vitro and in vivo.

MATERIALS AND METHODS

Preparation of BTP and Isolation of Theaflavins from Black-Tea Leaves. Dried black-tea leaves (250 g) were immersed in 5 L of 40% ethanol. The solution was applied onto 5 L of hydrophilic vinylpolymer (Toyopearl HW-40EC; Tosoh, Tokyo, Japan); subsequently, BTP was eluted using 50 L of 40% ethanol, followed by elution using 25 L of 70% acetone. Caffeine was largely eluted in the 40% ethanol fraction. The

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acetone fraction was evaporated, lyophilized, and used as the BTP fraction. Catechins, theaflavins, and caffeine contents in the BTP were analyzed by high-performance liquid chromatography (HPLC) with UV detection at 280 nm. Separation was achieved by using a 4.6 mm × 150 mm (3.5 μm particle size) Xbridge Shield RP18 column (Waters, Milford, MA) that was maintained at 40 °C. 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 Pure Chemicals (Osaka, Japan) and Nagara Science (Gifu, Japan), respectively. Total polyphenols were measured by using the Folin−Denis method (*27*). BTP contained 1.93% of catechins, 26.3% of theaflavins (TF, 5.69%; TF3G, 9.07%; TF3′G, 5.29%; and TFDG, 6.24%) and 84.5% of total polyphenols. Caffeine was not detected in the BTP fraction. To obtain sufficient amounts of pure TF, TF3G, TF3′G, and TFDG for in vitro study, a part of the BTP fraction was then subjected to a Wakosil-II 5C18HG column (20 mm × 250 mm: Wako Pure Chemicals) with UV detection at 280 nm. The solvent comprised 23% acetonitrile, 3% ethyl acetate, 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 similar to those reported previously (*28*). The purities of all of these theaflavins were found to be >96%. Epigallocatechin gallate (EGCG) and epicatechin gallate (ECG) were obtained from Wako Pure Chemicals. The purities of all of these catechins was found to be >98%.

Postprandial Hypertriacylglycerolemia in Rats. Male Wistar rats (8 weeks old; SPF; Japan SLC, Inc., Shizuoka, Japan) were fed a commercial nonpurified diet (CE-2; CLEA Japan, Inc., Tokyo, Japan) for a week and starved overnight before the administration of BTP. Next, blood was withdrawn from the jugular vein at 0 time; subsequently, 100 or 200 mg/kg of body weight of BTP dissolved in deionized water containing 0.2% of carboxymethylcellulose was orally administered at a concentration of 5 mL/kg of body weight. BTP was dissolved in carboxymethylcellulose to disperse them in the solution. Control group was administered deionized water. Immediately after the administration of BTP or deionized water, a lipid emulsion containing 200 g/L of soybean oil, 12 g/L of egg lecithin, and 22.5 g/L of glycerin was orally administered at a dose of 10 mL/kg (*29*). After the administration of the lipid emulsion, blood (100 μL) was withdrawn at 1, 2, 3, 4, 6, and 8 h via the jugular vein. Triacylglycerol levels in the serum that was separated from the blood were measured using a commercial kit (Triglyceride E test; Wako Pure Chemicals).

Lymphatic Recovery of ^{14}C-Trioleoylglycerol in Rats Cannulated in the Thoracic Duct. Before cannulation, 8-week-old male Sprague−Dawley (SD) rats (CREA Japan, Inc.) were fed a commercial chow for 1 week. The left thoracic lymphatic duct cephalad up to the cisterna chyli was cannulated after the rats had been anesthetized with nembutal (Dainippon Sumitomo Pharma Co., Ltd., Osaka, Japan), as described previously (*10*). A second indwelling catheter was placed in the stomach for administration of the test emulsion. After surgery, the animals were placed in restraining cages, and a solution containing 139 mM glucose and 85 mM 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 ^{14}C-trioleoylglycerol (PerkinElmer Inc., Wellesley, MA) with or without BTP. 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, 200 mg of trioleoylglycerol (Sigma, St. Louis, MO), 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 collected in ice-chilled tubes containing ethylenediaminetetraacetic acid (EDTA), and radioactivity was measured. At the end of the study, rats were killed by injecting an excess of nembutal (Dainippon Sumitomo Pharma Co., Ltd.). All of 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 Central Research Institute, ITO EN, Ltd., and Law 105 and Notification 6 of the government of Japan.

Activity of Pancreatic Lipase in Vitro. The activity of pancreatic lipase was measured according to the method of Han et al. (*30*). An emulsion (9 mL) containing 80 mg of trioleoylglycerol, 10 mg of phosphatidylcholine, and 5 mg of sodium taurocholate was mixed with 0.1 mol/L *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES) buffer (pH 7.0) containing 0.1 mol/L of sodium chloride prepared by sonication and maintained at 37 °C. A total of 100 μL of the emulsion solubilized in 0.1 mol/L of TES buffer containing 0.1 mol/L of sodium chloride (50 μL) and various amounts of BTP, theaflavin, and catechin solution (100 μL) was incubated with 5 U of porcine pancreatic lipase (Sigma) at 37 °C for 30 min. Released fatty acids were extracted using chloroform/heptane/methanol (49:49:2, v/v/v) and measured colorimetrically. The IC_{50} value of the BTP was obtained from the least-squares regression line of the plots of the logarithm of the BTP concentration (log) versus the inhibitory activity on pancreatic lipase.

Micellar Solubility of Hydrolysis Products of Triacylglycerol in Vitro. A micellar solution containing 1 mmol/L of oleic acid (Sigma), 0.5 mmol/L of 1-monooleoylglycerol (Sigma), 6.6 mmol/L of sodium taurocholate, 0.6 mmol/L of egg phosphatidylcholine (Sigma), and 132 mmol/L of sodium chloride in 15 mmol/L of sodium phosphate buffer (pH 6.8) was prepared by sonication and was maintained at 37 °C for 24 h to stabilize the micelles. A solution of BTP was added to the micelles (final concentration = 2 mg/mL of micelles) and incubated for 1 h at 37 °C. The micellar solution was passed through a 0.2 μm syringe filter (25 mm; GDD/X; Whatman Inc., Clifton, NJ). After lipid extraction, the concentration of total fatty acids in the filtrate was measured by GLC (*31*).

Statistical Analysis. Data were analyzed by the Tukey−Kramer test and the Dunnet test or by the Student *t* test to evaluate significant differences between pairs of means. Differences were considered to be significant at $P < 0.05$.

RESULTS

Postprandial Hypertriacylglycerolemia in Rats. The serum triacylglycerol concentrations at 1 and 2 h after BTP administration were significantly lower in both the BTP groups than in the control group (**Figure 1**). Although serum triacylglycerol concentration increased after the administration of a fat emulsion and reached the highest level at 2 h after the administration of the emulsion in the control and low-BTP groups, that in the high-BTP group remained at almost basal levels throughout the 8 h study period (**Figure 1**).

Lymphatic Recovery of ^{14}C-Trioleoylglycerol in Rats Cannulated in the Thoracic Duct. Lymph-flow rates were linear after cannulation, and there was no difference in the rates between the control and the BTP groups (151 ± 17 and 134 ± 8 mL/24 h, respectively). Lymphatic recovery of the radioactive ^{14}C-trioleoylglycerol at 1, 2, 3, 4, 5, 6, 8, and 24 h after BTP administration was significantly lower in the BTP group than in the control group (**Figure 2**).

Pancreatic Lipase Activity in Vitro. The pancreatic lipase activity was dose-dependently inhibited by the addition of BTP (IC_{50} = 0.254 mg/mL) (**Figure 3**). At concentrations of 0.5 and 1 mmol/mL, theaflavins with galloyl moieties (TF3G, TF3′G, and TFDG) inhibited the activity of pancreatic lipase (**Figure 4**), whereas theaflavins without galloyl moiety did not. At concentrations of 0.5 and 1 mmol/mL, the effectiveness of TFDG to inhibit the activity of pancreatic lipase was higher than that of EGCG, ECG, and the mixture of EGCG and ECG (**Figure 5**). BTP and TFDG exhibited the same inhibitory effect on the activity of pancreatic lipase when the total polyphenols was adjusted to the same amount as was present when BTP was added at concentrations of 0.25, 0.5, and 1 mg/mL (**Figure 6**).

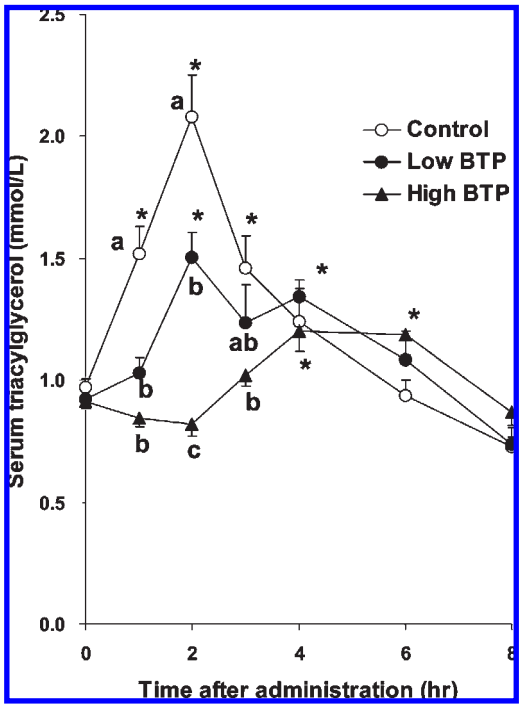

Figure 1. Effect of BTP on postprandial hypertriacylglycerolemia in rats intragastrically administered fat emulsions: ○, control group; ●, low-BTP group; ▲, high-BTP group. Data are means ± SE of 10 rats. The low-BTP group was orally administered a solution containing 100 mg/kg of body weight of BTP. The high-BTP group was orally administered a solution containing 200 mg/kg of body weight of BTP. Means not sharing a common letter at a time point significantly differ at $P < 0.05$. ∗, within a group, means significantly differ from those at time 0 at $P < 0.05$.

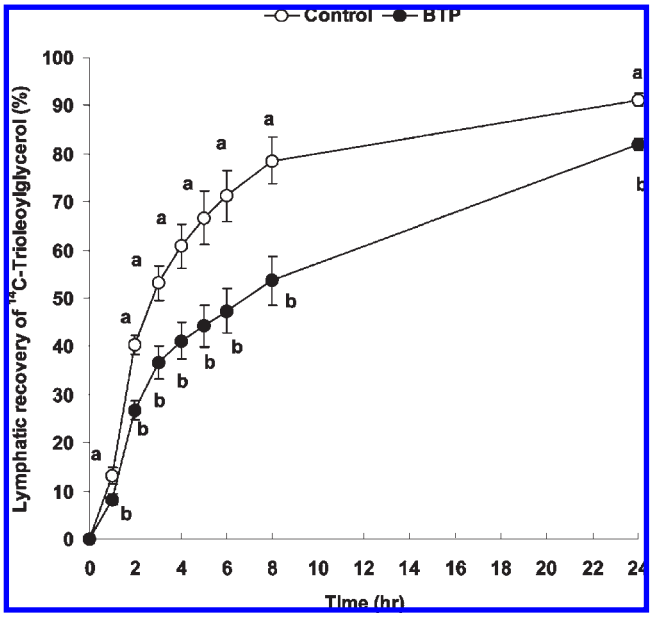

Figure 2. Effect of BTP on lymphatic recovery of [4-^{14}C]trioleoylglycerol in rats intragastrically administered a fat emulsion: ○, control group; ●, BTP group. BTP was intragastrically administered at 50 mg/rat together with a fat emulsion at 3 mL/rat. Data are means ± SE of seven rats. Means not sharing a common letter at a time point significantly differ at $P < 0.05$.

Micellar Solubility of Hydrolysis Products of Triacylglycerol in Vitro. When bile salt micelles containing oleic acid, 1-monooleoylglycerol, and phosphatidylcholine were incubated with BTP

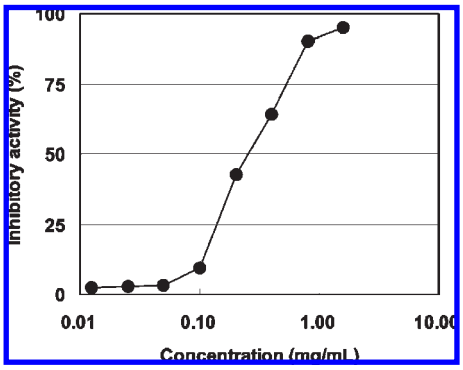

Figure 3. Inhibitory effect of BTP on pancreatic lipase. BTP was added at concentrations of 0, 0.0125, 0.025, 0.05, 0.1, 0.2, 0.4, 0.8, and 1.6 mg/mL. Data are means of triplicate experiments.

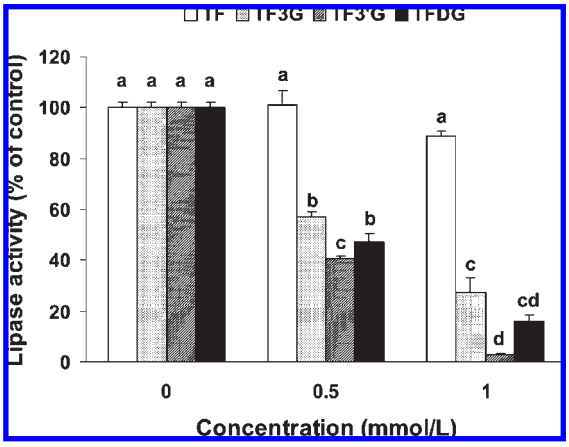

Figure 4. Effect of purified theaflavins on the activity of pancreatic lipase in vitro. The enzyme activity, that is, the amount of fatty acids released at 0 mmol/L of theaflavins, is estimated to be 100%. Purified theaflavins were added at concentrations of 0.5 and 1 mg/mL. Data are means ± SE of triplicate experiments. Means not sharing a common letter significantly differ at $P < 0.05$.

in vitro, precipitation was not observed; the concentration of total fatty acids originating from oleic acid, monooleoylglycerol, and phosphatidylcholine in the bile salt micelles was not affected by the addition of BTP (data not shown).

DISCUSSION

This is the first report of BTP-mediated suppression of postprandial hypertriacylglycerolemia in rats (**Figure 1**). Because lymphatic recovery of trioleoylglycerol was suppressed by the administration of BTP (**Figure 2**), it might be considered that at least the major cause of BTP-mediated suppression of postprandial hypertriacylglycerolemia may be the suppression of triacylglycerol absorption in the intestine. BTP dose-dependently inhibited the activity of pancreatic lipase in vitro (**Figure 3**). Addition of BTP to a bile-salt micellar solution containing the hydrolysis products of triacylglycerols, fatty acids, and monooleoylglycerol did not affect the total fatty acid content in the micelles (data not shown). This shows that BTP do not exclude oleic acid and monooleoylglycerol from the bile-salt micellar solution, suggesting that they do not suppress triacylglycerol absorption by inhibiting micellar solubility of hydrolysis products of triacylglycerols. These conclusions strongly suggest that the suppression of the transport of trioleoylglycerols to the lymph is caused by the inhibition of pancreatic lipase. However, in the

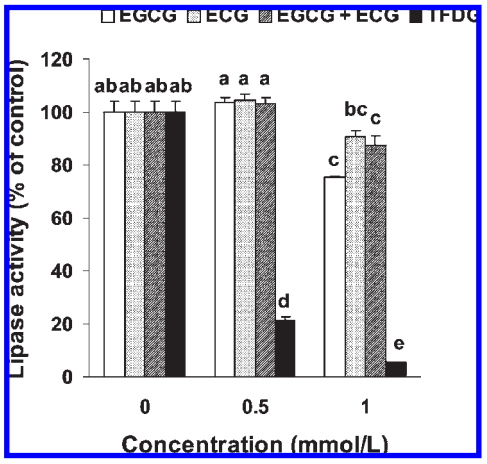

Figure 5. Effect of EGCG, ECG, a mixture of EGCG and ECG, and TFDG on the activity of pancreatic lipase in vitro. The enzyme activity, that is, the amount of fatty acids released at 0 mmol/L of EGCG, ECG, mixture of EGCG and ECG, and TFDG, is estimated to be 100%. For the mixture of EGCG and ECG, EGCG and ECG were mixed in equimolar amounts and total molar concentration was adjusted to 0.5 or 1.0 mmol/L. Data are means ± SE of triplicate experiments. Means not sharing a common letter significantly differ at $P < 0.05$.

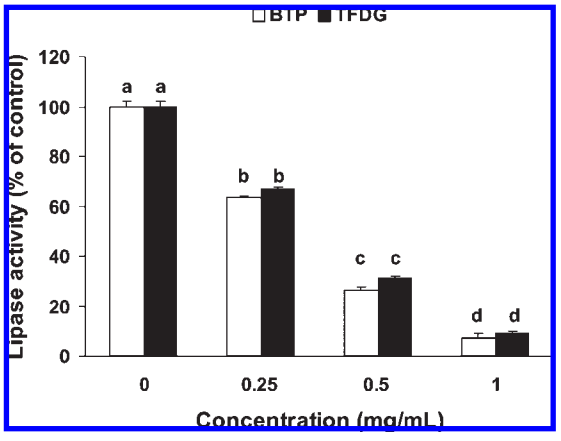

Figure 6. Effect of BTP and TFDG on the activity of pancreatic lipase in vitro. The enzyme activity, that is, the amount of fatty acids released at 0 mg/mL of BTP and TFDG, is estimated to be 100%. BTP was added at concentrations of 0.25, 0.5, and 1 mg/mL. Because BTP contained 84.5% of total polyphenols, TFDG was added at concentrations of 0.21125, 0.4225, and 0.845 mg/mL to obtain similar levels of total polyphenols. Data are means ± SE of triplicate experiments. Means not sharing a common letter significantly differ at $P < 0.05$.

present study, whether BTP inhibited pancreatic lipase in vivo was not evident. Nonetheless, because our study demonstrated that BTP administration simultaneously suppressed postprandial hypertriacylglycerolemia and lymphatic recovery of triacylglycerols, it seems reasonable to consider that BTP might inhibit pancreatic lipase activity in vivo as well. There is a possibility that BTP might directly bind to dietary fat—in the stomach or in the gut—thereby decreasing its absorption. This point should be studied.

In the present study, TF3G, TF3′G, and TFDG, which have galloyl moieties, inhibited the activity of pancreatic lipase in a dose-dependent manner, but not TF, which does not contain the galloyl moiety (**Figure 4**). Nakai et al. (*19*) showed that TF, TF3′G, and TFDG have the same inhibitory effect on the activity of pancreatic lipase. A reason for the discrepancy between our result and that obtained by Nakai et al. (*19*) might be due to the difference in the substrates used for pancreatic lipase. Nakai et al. (*19*) used 4-methylumbelliferyl oleate, which is a water-soluble substrate, and measured the amount of 4-methylumbelliferone released by lipase. On the other hand, we used trioleoylglycerol as a substrate in a lipid emulsion and measured the amount of fatty acids released by lipase. Shishikura et al. (*32*) showed that freeze-dried black-tea powder increased the mean droplet size of olive oil emulsion and decreased its specific surface area. It has been reported that the activity of pancreatic lipase is affected by the interface properties of the emulsion (*33*). Therefore, the results obtained by our method, that is, by using trioleoylglycerol as a substrate in a lipid emulsion, are thought to reflect both the direct inhibitory activity on the enzyme or substrate and the action on the interface properties of the emulsion. On the other hand, the method involving the use of 4-methylumbelliferyl oleate as a water-soluble substrate is thought to reflect only direct inhibitory activity on the enzyme or substrate. It is well-known that in the intestine, dietary fat is digested by pancreatic lipase present on the surface of the lipid emulsion. We believe that our experimental method is more appropriate for measuring the pancreatic lipase activity.

BTP contain 84.5% of total polyphenols, including unknown polyphenols besides catechins and theaflavins. It is possible that such unknown polyphenols in BTP also influence the activity of pancreatic lipase. In the present study, 0.5 and 1 mmol/mL of TFDG were shown to be more effective in inhibiting the activity of pancreatic lipase than a mixture of EGCG and ECG at the same concentrations (**Figure 5**). BTP and TFDG exhibited the same inhibitory activity on pancreatic lipase when the amount of total polyphenols was adjusted to the same level as that when BTP was added at concentrations of 0.25, 0.5, and 1 mg/mL (**Figure 6**). These results suggest that unknown polyphenols in BTP influence the activity of pancreatic lipase in a similar manner. Kusano et al. (*32*) reported that polymer-like oxidation products in black tea have inhibitory activity on pancreatic lipase. This finding supports our observation.

Investigations 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 (*35*). Because information on the absorption and metabolism of BTP and theaflavins is very limited (*36*), more studies on the safety, absorption, and metabolism of these compounds should be performed.

In the present study, we administered relatively high amounts of black-tea components to rats (**Figure 1**) in order to obtain a conspicuous difference. We consider that the effective dose of BTP in humans can be lower than that used in rats. We previously showed that green-tea catechins suppress postprandial hypertriacylglycerolemia in rats (*10*). In that study, we administered 100 mg/kg of body weight of green-tea catechins to rats to observe the suppression of postprandial hypertriacylglycerolemia. However, in a human study, we showed that the catechins suppressed postprandial hypertriacylglycerolemia at 215 mg/capita in humans (*25*). Therefore, we expect that BTP in a reasonable amount might also be able to suppress postprandial hypertriacylglycerolemia in humans.

In conclusion, our study suggests that BTP suppress postprandial hypertriacylglycerolemia—a risk factor for the development of coronary heart disease. It has been observed that BTP have a cholesterol-lowering activity in humans (*21*, *35*) and a preventive activity on low-density lipid (LDL) oxidation in

vitro (*22*). Therefore, BTP may be utilized as food components to prevent coronary heart disease.

ABBREVIATIONS USED

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

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