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Crude Cacao *Theobroma cacao* Extract Reduces Mutagenicity Induced by Benzo[a]pyrene Through Inhibition of CYP1A Activity *In Vitro*

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Polyphenols have been shown to have potent antioxidant activity, and therefore, food containing polyphenols is expected to contribute to the prevention of cancer. However, food contains not only polyphenols but also various other constituents. We used the Ames test to investigate the effects of crude extracts of whole cacao products, which are known to be rich in polyphenols, on the mutagenicity of benzo[a]pyrene (B[a]P) in *Salmonella typhimurium* strain TA 98 and tert-butyl hydroperoxide (t-BuOOH) in *S. typhimurium* strain TA 102. B[a]P induces mutagenicity by metabolic activation and t-BuOOH induces it by generation of free radicals. While white chocolate did not modulate the numbers of revertant colonies produced by B[a]P treatment, milk chocolate and cacao powder extracts did. On the other hand, surprisingly, none of the cacao products tested affected the number of revertant colonies when t-BuOOH was used as the mutagen. At maximum concentration (13.25 mg cacao powder/ml), the crude cacao powder extract reduced ethoxyresorufin O-deethylase activity to 17.4% of the control, suggesting that whole cacao products inhibit cytochrome P450 (CYP) 1A activity. In conclusion, inhibition of CYP1A activity by cacao products may prevent DNA damage by reducing metabolic activation of carcinogens. **Copyright © 2009 John Wiley & Sons, Ltd.**

Keywords: cacao; polyphenol; antimutagenic effect; radical scavenging; metabolic activation; cytochrome P450.

INTRODUCTION

Recently, the effects of polyphenols in food on biological function have been spotlighted. Cacao, red wine, and tea are well known to be rich in polyphenols, which are reported to reduce the risk of carcinoma (Jang *et al.*, 1997; Yamagishi *et al.*, 2003; Manna *et al.*, 2006). Many studies demonstrate the antimutagenic effect of polyphenols in vegetables and fruit (Yamada and Tomita, 1994; Yen and Chen, 1995). Moreover, cacao polyphenols are expected to contribute to the prevention of carcinogenesis due to their powerful effect as antioxidants (Vinson *et al.*, 2006). Scavenging of reactive oxygen species (ROS) by polyphenols is considered to be the major mechanism of their antimutagenic effects.

Numerous procarcinogens are catalyzed by metabolizing enzymes to either biologically inactive metabolites or to chemically reactive electrophilic metabolites that covalently bind to DNA and produce cancer (Conney, 2003). Mutagenic and carcinogenic chemicals, e.g., polycyclic aromatic hydrocarbons (PAHs) and heterocyclic aromatic amines (HCAs), require metabolic activation by the cytochrome P450 (CYP) superfamily, notably the CYP1A subfamily (Nebert, 1991), to electrophilic intermediates that can damage DNA. Increased CYP1A1 activity has been shown to be related to a high risk of lung cancer and colorectal cancer (McLemore *et al.*, 1990; Sivaraman *et al.*, 1994). CYP1A2, one of the major constitutively expressed CYP isoforms that contributes mainly to the metabolism of xenobiotics, also activates numerous procarcinogens to carcinogens. It is reported that CYP1A2 plays a role in human tobacco-related cancers (Smith *et al.*, 1996). Therefore, the activity of the CYP1A subfamily appears to be one of the most important factors in cancer initiation.

It is reported that some polyphenols modulate the activities of the CYP family and phase II-conjugating enzymes in drug metabolism (Young *et al.*, 2006). Flavones and flavonols strongly inhibit CYP1A activity (Kanazawa *et al.*, 1998). Tea polyphenols are reported to have conflicting effects (inhibition and enhancement) on CYP1A1 activity (Anger *et al.*, 2005). Cacao mass contains the polyphenols clovamide, quercetin, epicatechin, catechin, and other constituents. In particular, epicatechin, catechin, and quercetin are abundant in cacao mass. It is reported that quercetin induces the expression of CYP1A1 via the arylhydrocarbon receptor (AhR) (Uda *et al.*, 1997; Ciolino *et al.*, 1999). Moreover, quercetin and catechin are known inhibitors of CYP1A-dependent metabolism (Tsyrlov *et al.*, 1994; Anger *et al.*, 2005; Ghazali and Waring, 1999). Thus, in addition to scavenging ROS, polyphenols show complex activities including the modulation of CYP-dependent metabolism, leading to antimutagenicity. Therefore, the anticarcinogenic effects observed in epidemiological studies of food containing multiple constituents, including polyphenols, show the combined effects of the bioactive

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constituents. In order to clarify the anticarcinogenic effects of thise food, we need to examine not only the individual effects of each bioactive constituent but also the combined effects of various components in food.

It is reported that cacao liquor polyphenols have antimutagenic effects against HCAs in *in vitro* and *in vivo* mutagenicity tests (Yamagishi *et al.*, 2000). Thus, chocolate and cocoa, typical cacao mass products, are expected to have anticarcinogenic effects. Although the effects of a number of polyphenols in chocolate have been examined individually, however, there have been few reports that demonstrate the combined effects of the complex polyphenols and other constituents in chocolate. In addition to various polyphenols, chocolate and cocoa consist of sugar, milk, vegetable oil, an emulsifier, fragrance, and dietary fiber. In order to clarify the antimutagenic effect of a diet containing cacao mass, we investigated the effects of crude chocolate solutions and cacao powder extracts on the mutagenicity caused by B[a]P and t-BuOOH in the Ames test. As mentioned previously, cacao polyphenols show both a radical scavenging effect and modulation of CYP1A activity. In order to detect the antimutagenic effect associated with the CYP1A activity, we investigated the effects of chocolate solutions and cacao powder extracts on the mutagenicity induced by B[a]P. B[a]P is metabolized by CYP1A and transformed to forms that can bind DNA. The mutagenicity induced by B[a]P was detected in *Salmonella typhimurium* strain TA 98. In order to detect the antimutagenicity due to scavenging of radicals, we investigated the effects of chocolate solutions and cacao powder extracts on the mutagenicity induced by t-BuOOH. Hydroxyl radicals ($HO^{\bullet}$) are generated by t-BuOOH through reaction with Fe(II) and damage DNA. The mutagenicity caused by t-BuOOH was detected in *S. typhimurium* strain TA 102, which has an AT-rich hot spot in the DNA sequence and is highly sensitive to ROS. We also studied the effects of crude cacao powder extracts on ethoxyresorufin O-deethylase (EROD) activity, which reflects CYP1A activity.

MATERIALS AND METHODS

Chemicals. S9 cofactor NADPH, glucose 6-phosphate (G6P), and glucose 6-phosphate dehydrogenase (G6PDH) were purchased from Oriental Yeast Co., Ltd. (Tokyo, Japan), bovine serum from Sigma Chemical Co. (St Louis, MO, USA), and resorufin and ethoxyresorufin from Wako Chemical Co. (Tokyo, Japan).

Chocolate. Chocolate bars and cacao powder were obtained from commercial producers. The milk chocolate was from Lotte Co., Ltd (Tokyo, Japan), white chocolate from Morinaga Co., Ltd (Tokyo, Japan), and defatted cacao powder from Asahi Co., Ltd (Kanagawa, Japan). Information on the ingredient amounts in chocolate products was obtained from product information disclosed by manufacturers (Table 1). Each sample was prepared as follows.

Chocolate solutions: 1 g of each chocolate bar was mashed and added to 3 ml of dimethyl sulfoxide (DMSO). The chocolate mixture was vortexed for 10 min. The original chocolate solution was diluted with DMSO 10 and 100 times for the experiment (0.167, 1.67, and 16.7 mg chocolate/50 μl). The chocolate solution was stored at 4 °C.

Table 1. Ingredients of milk chocolate, white chocolate, and cacao powder

	1 g of chocolate or cacao powder		
Ingredient	milk chocolate	white chocolate	cacao powder
Calories (kcal)	5.6	5.9	2.6
Protein (mg)	74	75	280
Fat (mg)	340	410	10
Carbohydrate (mg)	556	491	522
Sodium (mg)	0.61	0.92	0.23

Cacao powder extract: Cacao powder contains 38.89 mg polyphenol per g. Therefore, 0.265 g of cacao powder was added to 1 ml of the solvent distilled water:acetone (1:1), (13.25 mg cacao powder/50 μl). The mixture contained 10 mg polyphenols in 1 ml as the maximum. The mixture was vortexed for 10 min and centrifuged at 860 g for 5 min. The extract was collected in a barrier filter with a 0.2 μm pore and centrifuged. Prepared solutions were diluted with 0.1 M potassium buffer to adjust the concentration of cacao powder.

Animals and treatments. Eight-week old male Wistar rats weighing 150–180 g (SLC, Hamamatsu, Japan), were housed at 24 ± 1 °C with a 12-h light and 12-h dark cycle, and given laboratory feed (MR stock, Nosan Co., Yokohama, Japan) and water *ad libitum* for one week. Rats were administered (i.p.) Sudan III, one of the potent inducers of CYP1A, at 40 mg/kg body weight for 3 days. The dose of Sudan III used was based on Fujita *et al.* (1984). Twenty-four hours after the last dose, rats were asphyxiated with carbon dioxide, and their livers were removed. All experiments using animals were performed under the supervision and approval of the Institutional Animal Care and Use Committee of Hokkaido University.

Preparation of cytosol and microsomes. Rat liver was perfused with ice-cold 0.1 M potassium phosphate buffer and homogenized. The homogenates were centrifuged at 9000 g for 20 min. The post-mitochondrial supernatants (S9 fraction) obtained were further centrifuged at 105 000 g for 60 min, and the supernatant was collected as the cytosol fraction. The microsomal pellet resulting from the second centrifugation was resuspended in 1.15% (w/v) KCl solution and centrifuged again for 60 min at 105 000 g for washing. The washed microsomal fractions were then suspended in 0.1 M potassium phosphate buffer (pH 7.4). All fractions were stored at –80 °C.

The protein concentration was determined by the method of Lowry *et al.* (1951), using bovine serum albumin as the standard.

Ames test. The Ames test was performed according to the preincubation method of Ames *et al.* (1975) using *S. typhimurium* strains TA 98 and TA 102, with minor modification as follows. Five-hundred microliters of the liver S9 incubation mixture containing the complete NADPH generating system and NADH or phosphate-buffered saline, 100 μl of overnight culture of TA 98 or TA 102, and 50 μl of chocolate solution or solvent alone

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were added to test tubes. Then, 50 µl of B[a]P (9.9 nmol) or t-BuOOH (1 µmol), as the mutagen, and DMSO (negative control for B[a]P) or 100 mM potassium phosphate buffer (negative control for t-BuOOH) were added, and the tubes were incubated for 25 min at 37 °C in a rotary water bath. Two ml of top agar was then added to every tube, vortexed, and the contents poured onto a minimum glucose medium plate. The plates were incubated for 48 h at 37 °C, and the number of revertant colonies was counted manually. We repeated the experiments at least twice.

The mutagenic response was considered to be positive when the mean number of revertant colonies was double that of the negative control. An antimutagenic effect was assessed if the number of colonies was fewer than half the number of the positive control and the reduction in the number of revertant colonies was obtained in dose-dependent manner (two-fold rule, Hamada *et al.*, 1994).

Ethoxyresorufin O-deethylase activity. EROD activity was determined by a fluorescence intensity assay using the general principles described by Crespi *et al.* (1997). The fluorescence of resorufin was detected with an excitation wavelength of 550 nm and emission wavelength of 590 nm. Reaction mixtures were 1 ml solution/tube containing 10 mM G6P, 10 mM $MgCl_2$, 500 µg microsomal protein, chocolate extract (various concentrations), and 10 µM ethoxyresorufin in 100 mM potassium phosphate buffer at pH 7.4. The tubes were preincubated in a dark room for 5 min at 37 °C, and the reaction was started by adding 20 µl of a mixture of 50 mM NADPH and 200 U G6PDH.

Although polyphenols are known to inhibit G6PDH activity in generating systems, the concentrations of polyphenols used were markedly lower than the IC50 of polyphenols in our study. Shin *et al.* (2008) reported that the IC50 µmol/L) of catechin and epicatechin for G6PDH activity were >>> 1000 µM. Indeed, the predicted concentrations of epicatechin were higher than 1000 µM (13.25 mg cacao powder/ml). However, we observed strong inhibitory effects of cacao powder extract on EROD activity at concentrations lower than 1000 µM (~10.6 mg cacao powder/ml) in preliminary experiments. In addition, the concentrations of NADPH 0.5 mM in this study were high enough for CYP-dependent reactions, because 0.25 mM was the optimal amount for the measurement of a CYP-dependent reaction without the G6PDH generating systems (Burke and Mayer, 1974).

Polyphenols are known to exhibit fluorescence. On the measurement, we prepared calibration mixtures containing various concentrations of chocolate extract and the same components as the reaction mixture except the microsomal protein. The calibration mixtures exhibited fluorescence derived only from the cacao extract. The fluorescence value of the calibration mixtures was subtracted from that of the corresponding reaction mixtures to estimate the fluorescence derived from resorufin. This experiment was repeated twice.

RESULTS

Cacao products reduced mutagenicity of B[a]P in Ames test. Figure 1A shows the effect of each chocolate solution on the number of revertant colonies induced by B[a]P in the Ames test using *S. typhimurium* TA 98. Milk chocolate inhibited the mutagenicity of B[a]P. The numbers of revertant colonies induced by B[a]P in combination with each dilution of the milk chocolate solution (0.167, 1.67, and 16.7 mg chocolate/plate) were 78.4%, 85.6%, and 41.8% of the control, respectively. In contrast, the numbers of revertant colonies induced by B[a]P combined with each dilution of white chocolate solution (0.167, 1.67, and 16.7 mg chocolate/plate) were 97.8%, 89.3% and 74.8% of the control, respectively, indicating no or little effect at these concentrations.

Figure 1B shows the effect of cacao powder extract on the number of revertant colonies induced by B[a]P treatment in the Ames test with TA 98. Cacao powder extract strongly inhibited the mutagenicity of B[a]P in a dose-dependent manner. The numbers of revertant colonies induced by B[a]P combined with each dilution of cacao powder extract (1.325, 2.65, 6.625, and 13.25 mg cacao powder/plate) were 91.2%, 78.5%, 48.2%, and 27.3% of the control, respectively.

Cacao products did not reduce mutagenicity of t-BuOOH in Ames test. Figure 2A shows the effect of each chocolate solution on the number of revertant colonies induced by t-BuOOH treatment in the Ames test using *S. typhimurium* TA 102. The numbers of revertant colonies induced by t-BuOOH in combination with each dilution of milk chocolate solution (0.167, 1.67, and 16.7 mg chocolate/plate) were 97.4%, 118.5%, and 81.1% of the control, respectively, and those of white chocolate were 95.5%, 96.5%, and 86.6% of the control, respectively. In conclusion, neither milk chocolate nor white chocolate changed the numbers of TA 102 revertants, indicating that these concentrations had no effect.

Figure 2B shows the effect of cacao powder extract on the number of revertant colonies induced by t-BuOOH treatment in the Ames test with TA 102. The numbers of revertant colonies induced by t-BuOOH in combination with each dilution of cacao powder extract (1.325, 2.65, 6.625, and 13.25 mg cacao powder/plate) were 100.8%, 92.9%, 94.8%, and 98.6% of the control, respectively. Thus, cacao powder extracts at these concentrations did not change the number of revertants.

Cacao powder extract inhibited CYP1A activity. Figure 3 shows the EROD activity of rat liver microsomes from rats treated with Sudan III using 10 µM ethoxyresorufin as a substrate. The EROD activities in the presence of each dilution of cacao powder extract (1.325, 2.65, 5.30, 7.95, 10.60, and 13.25 mg cacao powder/tube) were 79.4%, 74.1%, 74.6%, 43.0%, 28.3%, and 17.4% of the control, respectively. Cacao powder extract inhibited EROD activity in dose-dependent manner.

DISCUSSION

Chocolate and cocoa contain various constituents, including polyphenols. CYP1A activity has been reported to be enhanced or inhibited by polyphenols (Anger *et al.*, 2005; Kanazawa *et al.*, 1998; Zhai *et al.*,

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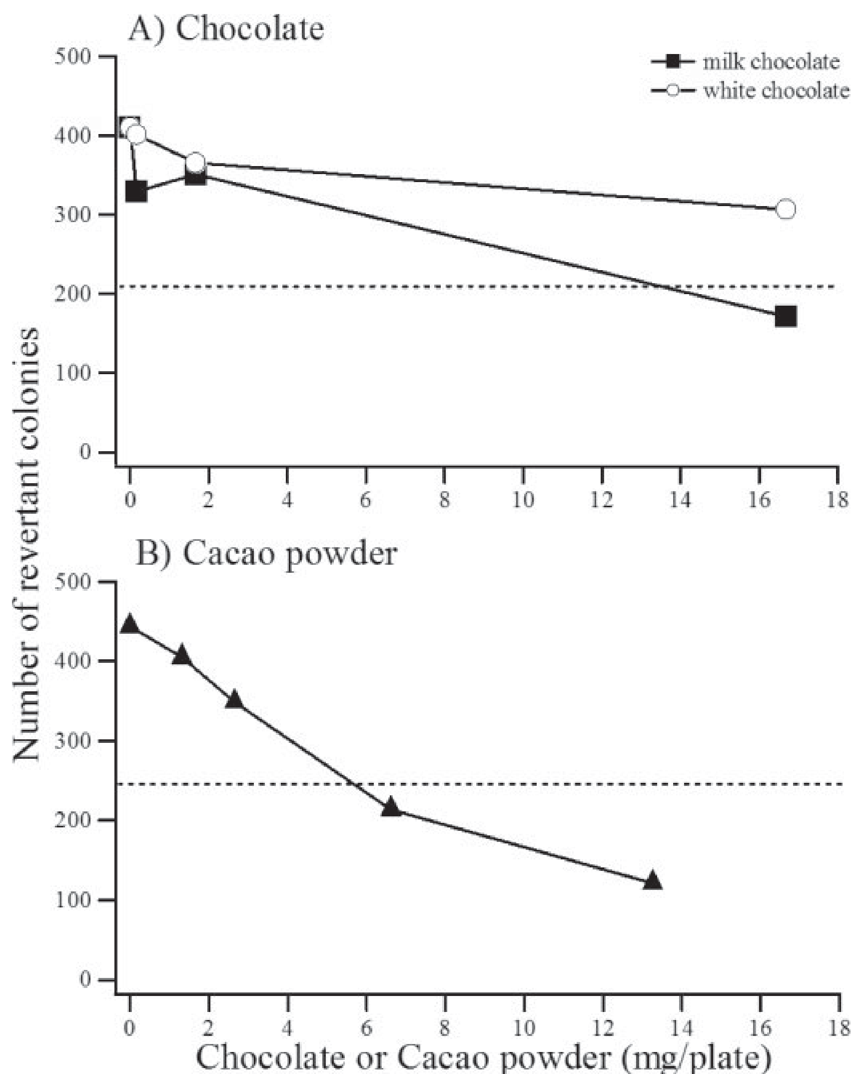

Figure 1. Effects of milk chocolate (filled square), white chocolate (opened circle), and cacao powder (filled triangle) on mutagenicity induced by B[a]P. The Ames preincubation assay was performed in the presence of the S9 fraction from the livers of Sudan III-treated rats using B[a]P as the mutagen and *S. typhimurium* strain TA 98 as the test strain with (A) each chocolate solution (0–16.7 mg chocolate/plate) or (B) cacao powder extract (0–13.25 cacao powder mg/plate) as described in the Materials and Methods section. The broken line represents half the number of TA 98 revertant colonies without chocolate solutions or cacao powder extract. Each value represents the mean of duplicate samples.

1998; Chen *et al.*, 2004). Thus, crude extracts of cacao mass products may have complex effects on CYP1A activity, and in fact, this study showed that the effects of whole cacao mass products on mutagenesis cannot be predicted by the effects of individual polyphenols alone.

The ingredients of white and milk chocolates were similar with the exception of cacao mass (Table 1). If any constituent, for example fat, sugar or milk, had reacted with any mutagenic agent, white chocolate might also have reduced the number of revertant colonies. However, white chocolate, which does not contain cacao mass, had no effect on the mutagenicity caused by B[a]P in the Ames test. In contrast, milk chocolate solution and cacao powder extract strongly reduced the mutagenesis of B[a]P in a dose-dependent manner. In addition, none of the cacao products showed an antimutagenic effect against t-BuOOH mutagenicity at these concentrations.

B[a]P is oxidized by CYP1A and transformed to activated forms (B[a]P-7-8-diol-9-10-epoxide and B[a]P-radical cation) that can damage DNA. In contrast, t-BuOOH generates hydroxyl radical through reaction with Fe(II) and damages DNA. Some cacao components, such as catechins, have been reported to reduce the mutagenicity of t-BuOOH due to their radical scavenging effects (Nikaido *et al.*, 2005). Thus, chocolate solutions or cacao powder extracts containing cacao polyphenols at concentrations higher than those used in our study may reduce the mutagenicity of t-BuOOH. In this study, we found that the reduction of the antimutagenic effects of cacao mass components was due to inhibition of CYP1A activity at low concentrations of the substrate. Kanazawa *et al.* (1998) reported the effects of epicatechin, catechin, and quercetin on the mutagenicity caused by 3-amino-1-methyl-5H-pyrido[4,3-b]indole (Trp-P-2), a food-derived carcinogenic heterocyclic amine, in the Ames mutagenic assay with *S. typhimurium* TA 98. Trp-P-2 expressed genotoxicity after metabolic activation by CYP1A, like B[a]P. They also showed that the most potent antimutagen, quercetin, did not have the potential to reduce the mutagenicity of direct mutagens, which do not need metabolic activation by CYP1A.

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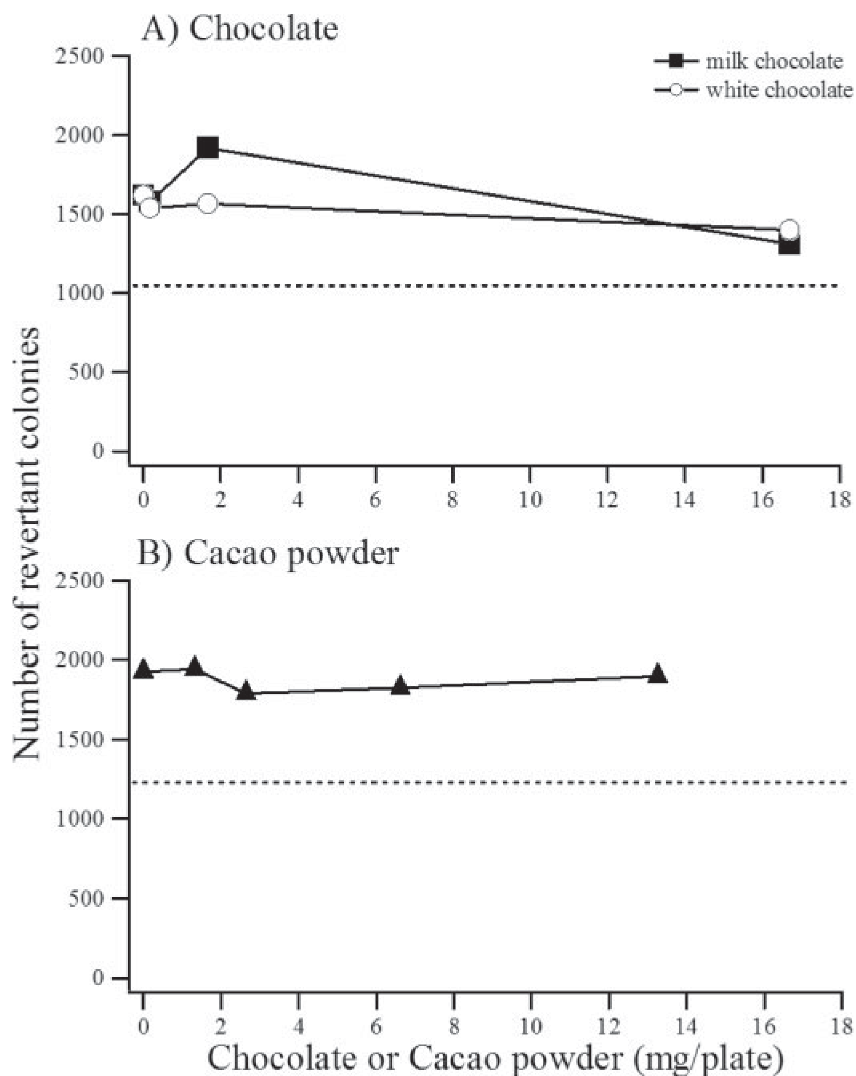

Figure 2. Effects of milk chocolate (filled square), white chocolate (opened circle), and cacao powder (filled triangle) on mutagenicity induced by t-BuOOH. The Ames preincubation assay was performed in the presence of the S9 fraction from livers of Sudan III-treated rats using t-BuOOH as the mutagen and *S. typhimurium* strain TA 102 as the test strain with (A) each chocolate solution (0–16.7 mg chocolate/plate) or (B) cacao powder extract (0–13.25 cacao powder mg/plate) as described in the MATERIALS AND METHODS section. The broken line represents half the number of TA 102 revertant colonies without chocolate solutions or cacao powder extract. Each value represents the mean of duplicate samples.

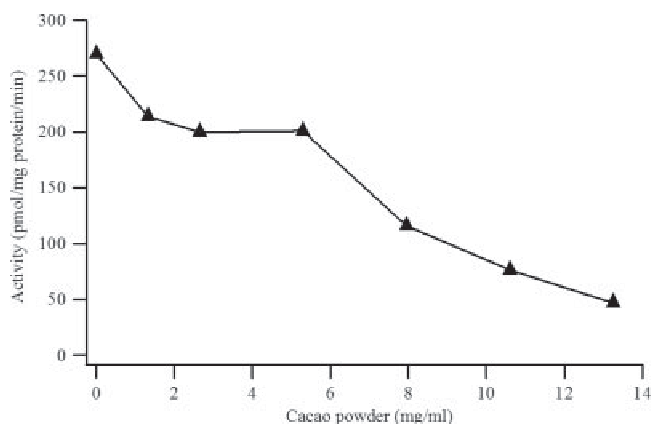

Figure 3. Effects of the concentration of cacao powder extract on EROD activity in liver microsomes from Sudan III-treated rats at 10 μM ethoxyresorufin. Details are described in MATERIALS AND METHODS. Each value represents the mean of duplicate samples.

EROD assays reflecting CYP1A activity indicated that cacao powder extract reduced CYP1A activity in a dose-dependent manner. These results demonstrated that the major mechanism of the antimutagenic effects of polyphenols in cacao mass may be inhibition of CYP-dependent metabolic activation of procarcinogens to carcinogens, but not radical scavenging.

The effect of cacao products on mutagenicity of Trp-P-2 was shown in an *ex vivo* Ames mutagenic assay (Yamagishi *et al.*, 2000). Yamagishi *et al.* (2000) injected TA 98 and Trp-P-2 into the mice 1 h after oral administration of cacao liquor extracts, and performed bacterial cultures using liver homogenate. They demonstrated that the numbers of Trp-P-2-dependent revertant colonies were significantly reduced by the co-treatment with cacao liquor extract (500 mg/kg body weight). While the physiological conditions are different from *in vitro* experiments, we suggest that the crude cacao might also reduce B[a]P induced mutagenicity *in vivo*.

Manach *et al.* (1999) revealed that quercetin and catechin were distributed mainly to the plasma and that

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quercetin and its metabolites were maintained longer than catechin in blood circulation. Although further study is needed, we suggest that these polyphenols in cacao mass may affect the CYP-dependent metabolic activation of mutagens in various organs, in addition to CYP in liver and intestine after ingestion.

In conclusion, our study revealed that extracts of cacao products containing various components show an antimutagenic effect on chemical mutagens that require metabolic activation by CYP1A *in vitro*. Cacao products may prevent initiation of cancer by inhibiting metabolic activation of carcinogens by CYP1A. However, whether the reported radical scavenging effects of some polyphenols contribute to anticarcinogenesis requires further study.

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