Original article

Camellia sinensis tea melanin suppresses transformation of the aryl hydrocarbon receptor and prevents against dioxin-induced toxicity in mice

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Summary The suppressive effects of *Camellia sinensis* tea melanin (CSTM) on transformation of aryl hydrocarbon receptor (AhR) induced by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) were disclosed for the first time. CSTM strongly inhibited TCDD-induced toxicity with IC_{50} equalling 20.4 μg mL^{-1}. Daily administration of CSTM (40 mg kg^{-1}, p.o.) prevented TCDD-induced body weight loss, ameliorated TCDD-induced mortality and prevented TCDD-induced hepatomegaly and thymic atrophy. Co-administration of CSTM significantly inhibited TCDD-induced hepatic CYP1 A1 activity. CSTM retarded transformation of AhR *in vitro*. In animals treated with CSTM, the antibody-secreting cells produced significantly ($P < 0.05$) more antibodies (32–34%) than the antigen control. Administration of TCDD caused a suppression of antibody-forming cells of 29–33% against the antigen control level. Co-administration of CSTM restored immunity to the control level. We demonstrated that CSTM directly competed with TCDD during the transformation of AhR and suppressed the downstream activation of genes associated with TCDD toxicity.

Keywords Aryl hydrocarbon receptor, *Camellia sinensis* tea, CYP1A1, melanin, 2, 3, 7, 8-tetrachlorodibenzo-*p*-dioxin.

Introduction

The 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) is one of the most toxic substances used as a reference standard for hazard and risk assessment of environmental and dietary contamination with dioxins (Ahlborg, 1994; De Vito & Birnbaum, 1994; Safe, 1994). Dioxins cause serious health problems because of their toxicities, lipophilicity and resistance to degradation (Tanabe *et al*., 1987; Webster & Commoner, 1994). The mechanism of dioxin toxicity is associated with binding to the cytosolic aryl hydrocarbon receptor (AhR) and subsequent transformation of the receptor (Whitlock, 1990; De Vito & Birnbaum, 1994; Denison *et al*., 1998). AhR is a ligand-activated transcription factor that exists in most cell and tissue types of the body (Pohjanvirta & Tuomisto, 1994). The transformed AhR complexes with dioxin receptor element (DRE) and activates downstream gene expression responsible for dioxin-correlated toxicity, including carcinogenicity and developmental, reproductive and immunological impairment (Silbergeld & Gasiewicz, 1989; Bock, 1994; Hankinson, 1995). Transformation of AhR is one of the first and key steps in the development of dioxin toxic effects. Therefore, suppression of AhR transformation is an important prerequisite in preventing dioxin-associated toxicity.

Natural flavonoids suppress the transformation of AhR (Ashida *et al*., 2000) and prevent activation of P450 isozyme, CYP1A1 (Ciolino & Yeh, 1999; Quadri *et al*., 2000). Green tea and its catechins also showed strong competition with TCDD for binding to AhR (Williams *et al*., 2000, 2004). These antagonistic properties were also found for polymeric polyphenols of low molecular catechins derived from black tea (Krishnan & Maru, 2004). However, scarce information is available about tea melanin derived from the *Camellia sinensis* tea leaves (Sava *et al*., 2001b). *Camellia sinensis* tea melanin (CSTM) represents the high molecular polyphenols possessing physicochemical characteristics

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of typical melanin. The analogous melanin pigments derived from other sources were intensively studied earlier (Nicolaus, 1968; Prota, 1998), and multiple biological activities were disclosed. Similarly, CSTM demonstrated a wide range of biochemical and pharmacological activities including antioxidant, free radical scavenging and immunostimulating effects (Sava *et al.*, 2001a; Hung *et al.*, 2002).

Recently, we have discovered that CSTM renders antagonistic effects against AhR *in vitro*. Our primary intention was to examine whether CSTM could work against toxicity of TCDD that is known as the most potent toxicant among dioxin-type chemicals. The previously disclosed antitoxic properties of CSTM were considered a major prerequisite in realisation of protective activity *in vivo*, especially in view of the immunostimulating effect of CSTM (Sava *et al.*, 2001a), which was found to be important for realisation of liver protection against acetaminophen toxicity (Hung *et al.*, 2004). It is likely that the immunostimulating properties of CSTM may be utilised in protection against TCDD toxicity, because such an effect as immunosuppression is associated with dioxin toxicity but does not require activation of AhR (Kerkvliet *et al.*, 1990). Thus, the purpose of this study was to investigate CSTM-inhibitory mechanisms, with or without AhR, against the adverse effects of TCDD.

Materials and methods

Materials

Camellia sinensis tea was manufactured in Miaoli, Taiwan. Tea leaves were identified in the Institute of Chinese Pharmaceutical Sciences, China medical University, and a voucher specimen (GSH-001) was deposited in the Herbarium of this Institute. TCDD was purchased from AccuStandard (New Haven, CT, USA). [^{32}P]-ATP was purchased from Amersham (Piscataway, NJ, USA). T4 polynucleotide kinase and Sephadex G-25 spin column were from Roche Diagnostics, Co. (Indianapolis, IN, USA). Ethoxyresorufin, resorufin, glucose-6-phosphate, glucose-6-phosphate dehydrogenase and NADPH (the reduced form of Nicotinamide Adenosine Dinucleotide Phosphate) were purchased from Sigma (St Louis, MO, USA). Other chemicals and solvents were of analytical grade or HPLC grade.

Extraction, fractionation and characterisation of *Camellia sinensis* tea melanin

Isolation of CSTM was performed according to a previous report (Sava *et al.*, 2001b). Monomeric polyphenols were removed by treatment of tea leaves with boiling water at volume ratio (solid/liquid) of 1:10 for 10 min followed by filtration. Solid matter obtained was immersed in water at a temperature of 40 °C at volume ratio 1:10 and pH 10.5 adjusted with 10% NH_4OH. Extraction time was reduced to 12 h to avoid excessive oxidation of CSTM. The extract was filtered and acidified with 2N HCl to pH 2.5 and then centrifuged at 15 000 × ***g*** for 15 min to form pellet. Acid hydrolysis of pellet matter was employed to remove carbohydrates and proteins (Harki *et al.*, 1996). Organic solvents (chloroform, ethyl acetate and ethanol) were used to remove lipids and related compounds. In addition, CSTM was reduced by treatment with Ti^{+3} (Horak & Gillette, 1971). The reduced CSTM was dialyzed against milli-Q water to remove Ti^{+3} from CSTM. Finally, the reduced sample was suspended in distilled water, and 0.1 M NaOH was added dropwise to dissolve CSTM and to adjust pH to 7.0. The solution was filtered through a Nalgene 0.45 μm filter. CSTM was fractioned on a Sephadex G-75 column (1.6 × 40 cm) at 0.5 mL min^{-1} flow rate of phosphate buffer (50 mM, pH 7.5). Fractions were monitored at 280 nm. All operations were conducted under N_2. Physical and chemical characteristics of CSTM were examined according to conventional procedures (Nicolaus, 1968; Paim *et al.*, 1990; Prota, 1998). Ultraviolet-visible (UV) absorption spectra were recorded on a JASCO V-530 UV-Visible Spectrophotometer. Infrared (IR) spectra were recorded on a Perkin Elmer (Waltham, MA, USA) spectrometer (Model 1600 FT). Thin layer chromatography (TLC) was performed using silica gel as the stationary phase and chloroform/ethyl acetate/formic acid (6:4:1) as the mobile phase (Montedoro *et al.*, 1992). CSTM was retained at the origin in TLC separation and did not produce any additional signs on the chromatogram. Comparisons with caffeine, catechin, epicatechin, epicatechin gallate, epigallocatechin, epigallocatechin gallate and theaflavin assured that CSTM preparation was free from these components.

Animals and treatment

Animal treatments were performed following 'The Guidelines for the Care and Use of Experimental Animals' of the institute. Eight-week-old female C57BL/6J mice were housed at room temperature of 22 ± 2 °C with 12 h light/dark cycle and fed standard rodent chow and water *ad libitum*. Mice were randomly assigned to four groups consisted of ten mice including control group (not receiving any treatment), negative control (receiving CSTM alone), positive control (received TCDD alone) and experimental group receiving both TCDD and CSTM. TCDD was administered in a single dose of 100 μg kg^{-1} body weight by oral gavage with corn oil as the vehicle on day 0. Four hours after TCDD treatment, CSTM was given by gavage once a day at a dose of 40 mg kg^{-1} body weight. After the initial treatment on day 0, CSTM was administered once a day

at the same dose for thirteen consecutive days. During the study, the body weights of all mice were measured before administration. Animals were killed at the end of experiment (15th day) by cervical dislocation. Liver, thymus and spleen were isolated, and organ weights of all mice were measured. Excised samples were washed with normal saline and stored at −70 °C for assays.

Evaluation of hepatic CYP1A1 activity

Livers were homogenised with Teflon-glass homogeniser in 3 mL of 0.15 M KCl. Microsomes were prepared by differential centrifugation (Wei *et al*., 1995). The microsomal pellet was resuspended in 100 mM potassium phosphate buffer (pH 7.25). Protein concentrations were determined with bicinchoninic acid (Smith *et al*., 1985). A fraction of microsomal suspension was reserved for Western blot analysis. Hepatic CYP1A1 activity was assayed using ethoxyresorufin-*O*-deethylase (EROD). The reaction mixture contained microsomal proteins, 50 mmol L^{-1} potassium phosphate (pH 7.4), 1.5 mol L^{-1} ethoxyresorufin and an NADPH regenerating system (4 mmo L^{-1} $MgCl_2{\cdot}6H_2O$, 5 mmol L^{-1} glucose-6-phosphate, 1 unit mL^{-1} glucose-6-phosphate dehydrogenase and 0.5 mmol L^{-1} NADPH). Reaction was initiated by adding NADPH and terminated after 10 min of incubation at 37 °C by adding 0.25 mL of 20% trichloroacetic acid. Activity was determined by measuring the formation of resorufin on spectrofluorometer (HITACHI F-3000; Hitachi, Tokyo, Japan) using an excitation wavelength of 530 nm and an emission wavelength of 585 nm. The results were reported as nmol min^{-1} mg^{-1} protein.

Western blot of CYP1A1

Twenty micrograms of microsomal protein was boiled for 5 min in loading buffer (6% SDS, 20% sucrose, 0.25M dithiothreitol and 0.1% bromphenol blue) and resolved on a 10% SDS-polyacrylamide Tris-glycine gel. The resolved proteins were transferred onto a nitrocellulose membrane following incubation with rabbit CYP1A1 primary antibody (Chemicon International, Inc., Temecula, CA, USA) for 1 h. The membrane was washed and incubated with horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature, and CYP1 A1 protein was detected with ECL™ Western blotting chemiluminescence system (Amersham Pharmacia Biotech, Piscataway, NJ, USA).

Evaluation of the antagonistic effects of *Camellia sinensis* tea melanin on aryl hydrocarbon receptor transformation

Transformation of AhR was determined using a DRE oligonucleotide probe containing a 26-bp AhR-binding site (Ashida *et al*., 2000). Synthetic oligonucleotides containing the complementary DRE-binding site (5′-GATCTGGCTCTTCTCACGCAACTCCG-3′ and 5′-GATCCGGAGTTGCGTGAGAAGAGCCA-3′) were annealed and radiolabelled with [^{32}P]-ATP using T4 polynucleotide kinase. Unincorporated nucleotides were removed from the labelled DRE probe on a Sephadex G-25 spin column. Cytosol fraction was obtained from the livers of six intact C57BL/6J mice. Livers were homogenised in buffer containing 25 mM Hepes, pH 7.5, 1 mM EDTA, 1 mM dithiothreitol and 10% v/v glycerol. Cellular debris was removed by centrifugation at 10 000 × ***g*** for 10 min at 4 °C. Clarified cytosol fraction was obtained by centrifugation at 100 000 × ***g*** for 60 min at 4 °C on ultracentrifuge Beckman L7-55. The amount of protein in the samples was determined using bicinchoninic acid (Smith *et al*., 1985) and bovine serum albumin as a standard. To estimate the antagonistic effects of CSTM on AhR transformation, the cytosolic fraction (4.0 mg protein per mL) was incubated with various concentrations of CSTM at 20 °C for 10 min and then with 1 nM TCDD dissolved in dimethyl sulfoxide (DMSO) or with DMSO (10 μL mL^{-1}) alone as a vehicle control (blank) for a further 2 h. The amount of 10 fmol [^{32}P]-labelled DRE probe was added, and the mixture was incubated for a further 15 min at room temperature. A 10 μL aliquot of each incubation mixture was loaded onto a 4% non-stacking native polyacrylamide gel prepared on TBE buffer (25 mM Tris, 22.5 mM borate, 0.25 mM EDTA) and was electrophoresed in the same buffer at 50 V for 1 h. After electrophoresis, the amount of [^{32}P]-DRE in the induced protein-DNA complex was determined using a Molecular Dynamics phosphorimager. The amount of transformed AhR bound with [^{32}P]-DRE oligonucleotide was calculated using quantity of radioactivity in protein-DNA complex minus that of blank sample. The amount of AhR inhibited by CSTM was expressed as fraction of AhR amount induced by TCDD alone.

Evaluation of *Camellia sinensis* tea melanin uptake

Hepatocytes were prepared from liver of intact mice using two-stage collagenase perfusion method described previously (Moldeus *et al*., 1978). The hapatocytes were suspended in Krebs–Henseleit buffer (pH 7.4), and cells were cultured in siliconised flasks at a cell density of 1×10^6 cells mL^{-1} at 37 °C in a humidified 5% CO_2, 95% air atmosphere. The viability of cell culture was maintained at 90–95%. The viability of hepatocytes was assessed by Tripan Blue exclusion method (Bergmeyer *et al*., 1965). To assay the CSTM uptake, the cell culture was divided into seven groups including control flask and flasks treated with different concentrations of CSTM: 0, 5, 10, 20, 30, 40 and 50 μg mL^{-1}. To evaluate the effect of TCDD on uptake of CSTM, an additional experiment was carried out in presence of 1 ng mL^{-1} of

TCDD. After 24-h incubation, the culture medium was aspirated; cells were washed in cold Krebs–Henseleit buffer (pH 7.4) followed by centrifugation several times until the supernatant was clear. Cells were collected and lysed by sonication in 1 mL of 10 mM KCl. Cellular debris was removed by centrifugation of the lysate at 10 000 rpm for 10 min, and the supernatant was transferred into a spectrophotometer cuvette. The spectral characterisation of melanin was performed with a Pharmacia LKB-Ultrospec III spectrophotometer. The concentration of CSTM was calculated from the calibration curve built at 500 nm.

Evaluation of antibody-forming responses

The experiment was performed on four separate groups of intact C57BL/6J mice consisting of four animals each. Mice received the same treatment with TCDD and CSTM or a combination of both as described above. On 15th day, the beginning of toxic exposure mice were injected into the tail vein with 1×10^8 sheep red blood cells (SRBC) prepared in 0.2 mL of saline. The untreated four animals received only SRBC (antigen control). Four days after their sensitisation with SRBC, the animals were killed, and their spleens were removed. Single splenocytes were prepared from each spleen in 5 mL of RPMI-1640 media. Enumeration of the antibody-forming cells (AFC) was performed using a plaque assay (Jerne & Nordin, 1963). AFC values were calculated for 10^6 splenocytes.

Statistical analysis

All data were presented as mean ± SEM. Group means for relative liver weights, EROD activity and AhR were evaluated using one-way analysis of variance (ANOVA). If the data were homogenous and normally distributed, multiple comparisons were made by Newman–Keuls test. If tests for normality or variance failed, the Kruskal–Wallis one-way ANOVA on ranks was carried out. The minimum level of significance was set at $P < 0.05$.

Results

Isolation, purification and characterisation of *Camellia sinensis* tea melanin

Camellia sinensis tea melanin was isolated from *Camellia sinensis* tea in accordance to previously reported protocol (Sava *et al*., 2001b) with minor adjustments. In particular, the preparation was treated with $TiCl_3$ as described earlier (Hung *et al*., 2002). Final preparation of CSTM contained 91% of 14 ± 3 kDa fraction that exhibited all principal properties common to natural melanin reported earlier (Nicolaus, 1968; Paim *et al*., 1990; Bilinska, 1996; Prota, 1998). Namely, the solution of CSTM in 0.1 M phosphate buffer (pH 8.0) exhibited optical spectrum similar to synthetic melanin. IR spectrum of CSTM also demonstrated similar structural peculiarities when compared with previously studied melanin pigments including synthetic melanin (Paim *et al*., 1990; Bilinska, 1996). Namely, the IR spectrum of CSTM showed a broad band at 3450 cm^{-1}, attributed to stretching vibrations of -OH and -NH_2 groups. A strong absorption at 1650 cm^{-1} was recognised as the vibrations of aromatic C = C or C = O groups. After the acid hydrolysis of CSTM, the intensity of both bands at 3450 and 1650 cm^{-1} was reduced, a phenomenon caused by the reaction between phenolic and carboxylic groups owing to the formation of lactones (Sava *et al*., 2001b), which likely is involved in various biological effects.

Thin-layer chromatography analysis suggested that CSTM isolated according to the mentioned procedure was not contaminated by any monomeric polyphenols. In addition, IR spectra showed poor resolution in the 'fingerprint region' (800–1600 cm^{-1}) when compared with monomeric catechins (Krishnan & Maru, 2004). These results evidenced that CSTM isolated from black tea and used for the present study was free from known biologically active components of black tea.

Protective effect of *Camellia sinensis* tea melanin against 2,3,7,8-tetrachlorodibenzo-*p*-dioxin toxicity

To study whether CSTM is able to reduce the 'wasting syndrome' elicited by TCDD, the different groups of C57BL/6J mice were exposed to TCDD and/or to CSTM. The changes in the body weight gain for different groups are shown in Fig. 1. At the end of experiment, the untreated animals on average gained 2.8 g in body weight (8.7% of initial weight of 32 g). CSTM treatment (40 mg kg^{-1}, p.o., daily) allowed the gaining of 1.3 g extra for control group. TCDD-treated mice (100 μg kg^{-1}., single p.o. administration) demonstrated loss of body weight. The significant loss started from day 5, followed by a marked suppression of body weight gain (Fig. 1). At the end of experiment, animals treated with TCDD lost 4 g (12.5%) on average when compared with control. Co-treatment mice with CSTM prevented TCDD-induced body weight loss. On 15th day of experiment, the average weight in the group of animals receiving both TCDD and CSTM was about the same as in control. During the experiment, no significant changes in feed intake were observed for any group of animals. The diet consumption in the control, CSTM, TCDD and CSTM + TCDD groups was 2.65 ± 0.12, 2.78 ± 0.15, 2.60 ± 0.22 and 2.91 ± 0.27 g day^{-1} per mouse, respectively.

The mortality caused by TCDD treatment was also reduced when animals received CSTM (Fig. 2). While four of ten mice died during experiment in the TCDD group, nine out of ten mice survived in the

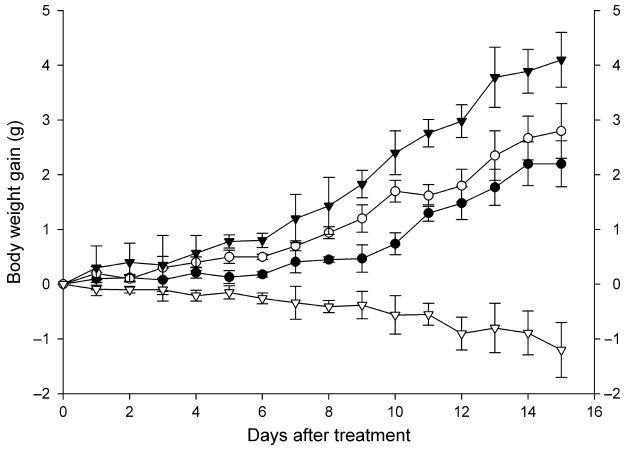

Figure 1 Protective effect of *Camellia sinensis* tea melanin (CSTM) (40 mg kg^{-1}, p.o., daily administration) on the body weight lost produced by single dose (100 μg kg^{-1}, p.o.) of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) on C57BL/6J mice. The values represent mean ± SEM of survived mice. Open circles represent control; filled triangles depict CSTM administered alone; open triangles represent treatment with TCDD alone and filled circles show co-treatment CSTM + TCDD.

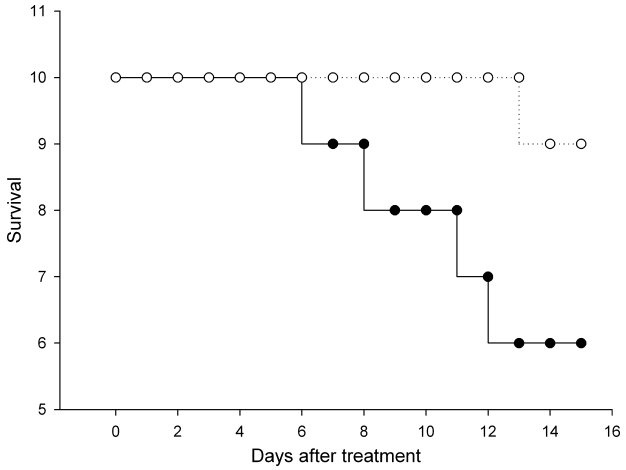

Figure 2 Survivalence of C57BL/6J mice treated with single dose of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) (100 μg kg^{-1}, p.o.). TCDD treatment depicted by filled circles and co-treatment with *Camellia sinensis* tea melanin (CSTM) (daily administration of 40 mg kg^{-1}, p.o.) showed by open circles. No mice died in control and in a group given CSTM alone.

CSTM + TCDD group. Both hepatomegaly and thymic atrophy were absent in TCDD + CSTM-treated mice. Thus, CSTM was able to rescue animals exposed to TCDD.

Effects of TCDD and CSTM on organ weight are shown in Table 1. Administration of CSTM alone caused no significant difference in liver weight when compared with control. For animals exposed to TCDD, the relative liver weight increased 1.5-fold. Co-treatment with CSTM significantly decreased manifestation of hepatomegaly. Relative weight of thymus was also decreased in mice poisoned with TCDD, but co-treatment with CSTM allowed recovery to normal weight of the organ. In addition, CSTM itself did not affect thymus weight, but single administration of TCDD caused decrease weight of spleen. Co-treatment with CSTM prevented loss of spleen weight without producing any harmful effect when administered alone.

Table 1 Changes in organ weights of C57BL/6J mice caused by oral administration of TCDD and CSTM

	Organ weights (% of body weight)		
Treatment	**Liver**	**Thymus**	**Spleen**
Control	5.1 ± 0.4	0.19 ± 0.02	0.3 ± 0.03
CSTM (50 mg kg^{-1})	5.9 ± 0.5	0.21 ± 0.02	0.34 ± 0.02
TCDD (100 μg kg^{-1})	7.8 ± 0.6*	0.05 ± 0.01*	0.22 ± 0.02*
TCDD + CSTM	5.9 ± 0.5	0.16 ± 0.02	0.27 ± 0.02

The values represent means ± SEM.
*Depict data significantly different from the control ($P < 0.05$). CSTM was administered daily in dose 40 mg kg^{-1} p.o. TCDD was given in dose 100 μg kg^{-1} body weight 4 h before the first dose of CSTM.

Effects on microsomal ethoxyresorufin-*O*-deethylase activity and expression of CYPA1 protein

The 2,3,7,8-tetrachlorodibenzo-*p*-dioxin-induced elevation of hepatic EROD activity while administration of

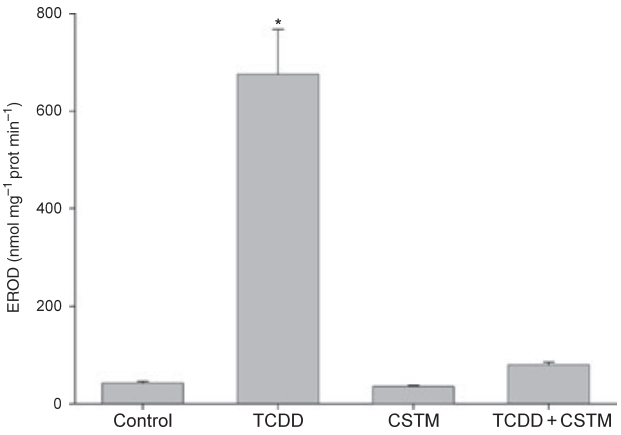

Figure 3 Effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and *Camellia sinensis* tea melanin (CSTM) on EROD activity in liver microsomes isolated from C57BL/6J mice. Treatments for each group are: control group, no treatment; TCDD, 100 μg kg^{-1}TCDD; CSTM, 40 mg kg^{-1} CSTM; and TCDD + CSTM, 100 μg kg^{-1} TCDD and 40 mg kg^{-1} CSTM. Data represent mean ± SEM for six animals. Asterisks depict results significantly different from the control ($P < 0.05$).

CSTM alone had no apparent effect (Fig. 3). Namely, EROD activity was increased in TCDD-treated mice by sixteen-fold over the control. Daily administration of CSTM did not elicit notable changes in hepatic EROD when compared with control. Treatment of TCDD + CSTM reduced EROD activity to a two-fold level of the control. Co-administration of CSTM with TCDD significantly inhibited activation of CYP1A1.

The profile of hepatic CYP1 A1 determined by Western blot (Fig. 4) resembled the profile of microsomal EROD activity (Fig. 3). In livers of untreated animals or CSTM group a very low level of CYP1A1 was observed. Administration of TCDD (100 mg kg^{-1}, p.o.) caused a twentyfold increase in protein level compared with control. Application of CSTM in addition to TCDD treatment decreased CYP1A1 amount to a 2.3-fold level above control. Apparently, reduction of CYP1A1 provided a possible explanation to the protective activity of CSTM against toxicity of TCDD. However, dose of CSTM has to be further increased to find out whether complete blockage of CYP1A1can be achieved.

Suppressive effect of *Camellia sinensis* tea melanin on aryl hydrocarbon receptor transformation

To investigate the possibility that CSTM might suppress AhR transformation, the cytosolic fraction derived from

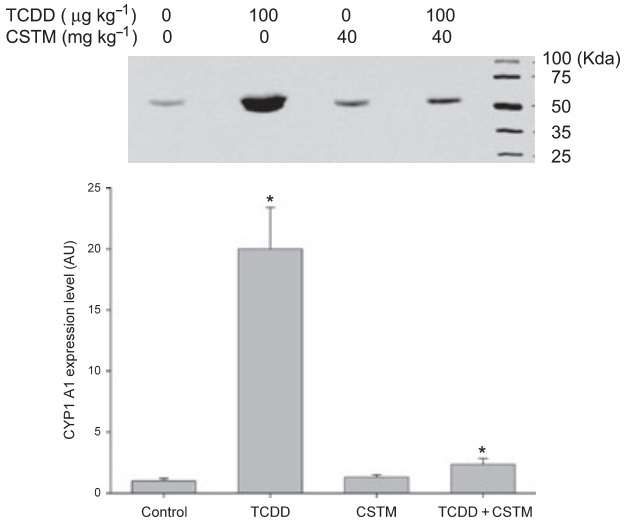

Figure 4 Expression of CYP1A1 in liver of C57BL/6J mice exposed to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and/or to *Camellia sinensis* tea melanin (CSTM). Upper panel represent the Western blot results. Lower panel represent expression of CYP1A1 protein (arbitrary units, AU) calculated from the density of Western blot bands. Treatments for each group are: control group, no treatment; TCDD, 100 μg kg^{-1} TCDD; CSTM, 40 mg kg^{-1} CSTM; and TCDD + CSTM, 100 μg kg^{-1} TCDD and 40 mg kg^{-1} CSTM. Data represent mean ± SEM for six animals. Asterisks depict results significantly different from the control ($P < 0.05$).

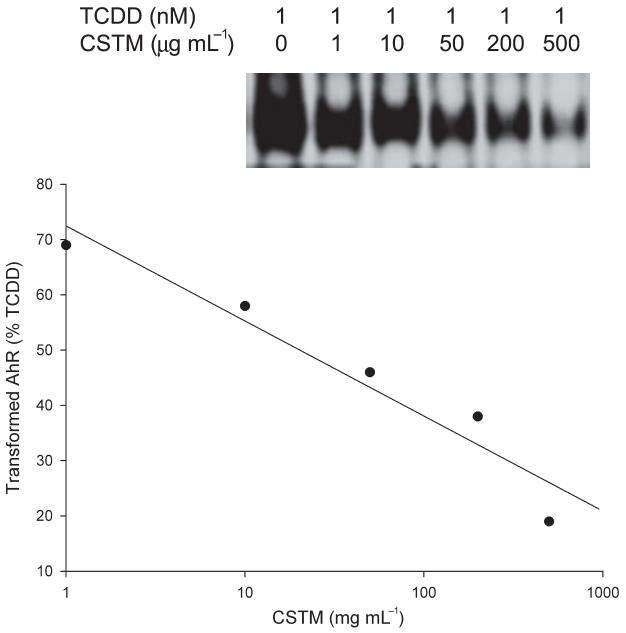

Figure 5 Suppressive effect of *Camellia sinensis* tea melanin (CSTM) on transformation of aryl hydrocarbon receptor (AhR). Upper panel is representative photograph obtained with Molecular Dynamics phosphorimager. Lower panel represent concentration-dependent effect of CSTM on the amount of AhR/dioxin receptor element (DRE) complex calculated in % to the initial amount this complex transformed with 1 nM of TCDD. Data presented are mean ± SEM obtained from six independent experiments plotted versus logarithm of CSTM concentration. The IC_{50} value calculated from the regression line at 50% of the level of transformed AhR/DRE.

intact C57B/L6 mice was incubated with different concentrations of CSTM in the presence of TCDD and labelled DRE-binding sequence. The resultant mixture was subjected to electrophoretic mobility shift assay. CSTM suppressed AhR transformation induced by 1 nM TCDD in a dose-dependent manner (Fig. 5, upper panel). To determine the 50% inhibitory concentration (IC_{50}), a logarithm of the concentration of CSTM against the ratio (R) of transformed AhR was plotted (Fig. 5), and the IC_{50} value was found from the regression equation

$$R = 72.47 - 17.19 \log[\text{CSTM}]$$

where R was expressed in % of the initial amount of AhR complex transformed by TCDD; CSTM concentration was expressed in μg mL^{-1}.

The calculated value of IC_{50} was 20.4 μg mL^{-1} suggesting a strong antagonistic effect of CSTM against TCDD. However, it was unclear whether the suppressive effect of CSTM on AhR transformation can be realised *in vivo*. To understand this possibility, we carried out the model experiment on cell culture of

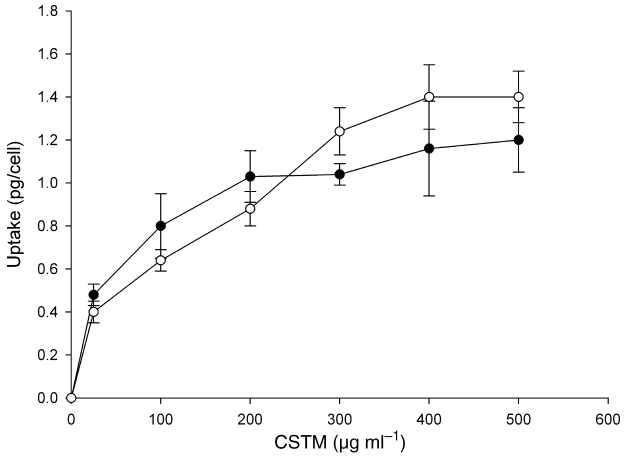

Figure 6 Intracellular uptake of *Camellia sinensis* tea melanin (CSTM) by hepatocytes after 24 h of incubation. Open circles represent measurements performed at presence of 1 ng mL^{-1} 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) in culture media. Filled circles represent CSTM alone (no TCDD added). Each point illustrates the mean value of six replications with SEM.

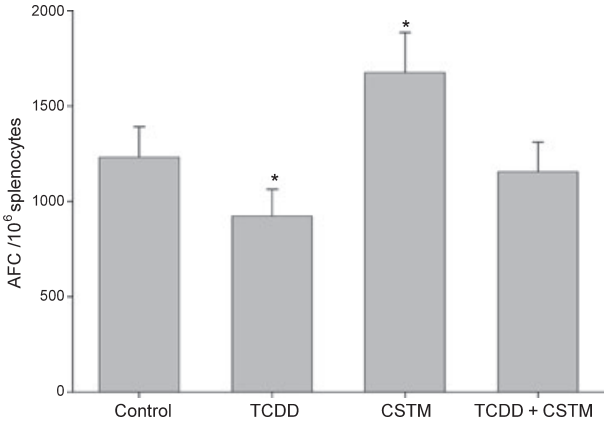

Figure 7 *In vivo* antibody-producing response caused by administration of single dose of TCDD (100 μg kg^{-1} p.o.) and daily dose of CSTM (40 mg kg^{-1}, p.o.) in female C57BL/6J mice. Results are mean ± SEM for four animals per treatment group. The asterisks depict the results significantly different from the control ($P < 0.05$). AFC means antibody forming cells.

hepatocytes allowing evaluative passing of CSTM through cell membrane. Spectrophotometric properties were utilised to determine the intracellular amount of CSTM. Intracellular concentration of CSTM increased following the increasing concentration of extracellular CSTM (Fig. 6). Intracellular concentration of CSTM reached the saturation level of 1.2 pg per one cell at concentration 50 μg mL^{-1} of extracellular CSTM. Thus, the uptake of CSTM was about 2.4% of the amount of extracellular CSTM. The uptake of CSTM was slightly affected by TCDD (1 nM). Within the concentration ranging from 0 to 22 μg mL^{-1}, the uptake of CSTM was suppressed by TCDD, but it was enhanced at higher concentrations. However, the differences (Fig. 6) were insignificant ($P > 0.05$).

Effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin and *Camellia sinensis* tea melanin on *in vivo* antibody-producing responses

Effect of TCDD and CSTM alone and the joint effect of CSTM + TCDDP on the humoral immunity of female C57BL6 mice were investigated. As expected, C57BL/6J mice exposed to TCDD alone at 100 μg kg^{-1} had suppressed plaque-forming cell (PFC) responses. In contrast, enhancement of the PFC response was observed in mice dosed with 50 mg CSTM per kg alone. Introduction of CSTM demonstrated an immunostimulating effect (Fig. 7). At doses of 100 mg kg^{-1} of CSTM, the antibody secreting cells produced significantly ($P < 0.05$) more antibodies (32–34%) than the antigen control. Administration of TCDD caused a suppression of AFC of 29–33% against the antigen control level. However, co-administration of CSTM restored immunity to the control level.

Discussion

Dioxins express toxicities through the transformation of AhR (Whitlock, 1990; De Vito & Birnbaum, 1994; Denison *et al.*, 1998). In the present study, CSTM, tea-derived melanin, showed a dose-dependent suppression of AhR (Fig. 5). Previous reports showed that green tea extract and its major compounds, catechins, suppressed AhR transformation and downstream *CYP1A* gene expression (Palermo *et al.*, 2003). However, the effects caused by CSTM were not associated with the presence of monomeric polyphenols in the preparation. Careful purification and analysis of CSTM indicated that our preparation was free from known biologically active components of black tea. Moreover, we tested several extracts obtained by shaking CSTM for 24 h with hexane, ethyl alcohol, acetone and ethyl acetate. As expected, no activities were found in the extracts obtained.

Evaluation of CSTM antagonistic effect gave an IC_{50} value of 20.8 μg mL^{-1}. Green tea extract showed an antagonistic effect on AhR transformation at concentration of about 250–500 μg mL^{-1} (Williams *et al.*, 2000). Thus, the effect of CSTM was much stronger than that of green tea extract. This extraordinary ability of CSTM is not clear. However, a similar phenomenon was reported earlier in respect to formation of microsome-catalyzed [3H]-B(a)P-derived DNA adduct with polymeric polyphenol of tea (Krishnan & Maru, 2004). This suggested that black tea polymeric polyphenols

caused relatively higher inhibitory effects than the parent monomeric compounds.

The understanding of CSTM antagonistic effect on AhR may be very important for utilisation of its protective activity against TCDD. As is well known, AhR is a ligand-dependent transcription factor that mediates the biological and toxic effects of different chemicals (Denison & Nagy, 2003). Binding ligands to AhR complex stimulates a conformation change in the AhR. In the nucleus, AhR dissociates from its associated protein subunits, and dimerisation with its nuclear protein partner aryl hydrocarbon receptor nuclear translocator (AhRNT) converts the AhR complex to its DNA-binding form (Chen & Perdew, 1994). Binding of the ligand:AhR:AhRNT complex to its specific DNA recognition site, DRE results in activation of the promoter and transcription of the gene (Denison *et al.*, 1998). The most responsive genes are those involved in xenobiotic metabolism, e.g. cytochrome P450 isozymes CYP1A1, CYP1A2 and CYP1B1.

In our experiments, we demonstrated (Fig. 3) that TCDD increased microsomal EROD in liver of C57BL/6J mice associated with activation of CYP1A1. Namely, CYP1A1 activity was increased in animals treated with 100 μg kg^{-1}, p.o. by sixteen-fold over control. However, co-administration of CSTM with TCDD significantly inhibited activation of this isozyme. A profile of hepatic CYP1A1 protein expression determined by Western blot (Fig. 4) was similar to the profile of microsomal EROD activity (Fig. 3). In livers of untreated animals or CSTM group the expression of CYP1A1 was very low. Administration of TCDD caused a twentyfold increase in protein expression level when compared with control. Application of CSTM in addition to TCDD treatment decreased the CYP1A1 amount to almost the control level. Thus, CSTM caused a strong inhibitory effect on CYP1A1. However, the dose of CSTM has to be further increased to find out whether complete blockage of CYP1A1 is possible.

It was unclear whether the suppressive effect of CSTM on AhR transformation can be realised *in vivo*. To understand this possibility, we carried out the model experiment on cell culture of hepatocytes allowing evaluative passing of CSTM through cell membrane. Such possibility was investigated before for synthetic melanin. For example, melanin has been reported to be actively phagocytised by PC12 cells (Offen *et al.*, 1999). In our present study, CSTM was added to the cell culture medium in concentrations ranging from 0 to 50 μg mL^{-1}. We have shown that the intracellular fraction of CSTM in cultured hepatocytes reached 2.4% of the extracellular concentration when 50 μg mL^{-1} was applied (Fig. 6). In gross estimate, the even distribution of CSTM in a body would give a concentration of 40 μg mL^{-1} at an *in vivo* dose of 40 mg kg^{-1}. This would allow an uptake of 1.1 pg per one cell of hepatocyte. Assuming that the volume of a single cell is about 5.10^{-6} μL, the intracellular concentration of CSTM would be around 220 μg μL^{-1}, sufficient to inhibit transformation of AhR on 70% (Fig. 5). Thus, a 40 mg kg^{-1} dose of CSTM seems to be insufficient for blocking the toxicity of TCDD. Future dose-effect studies are required to bring more clarity into this effect.

One of the most sensitive toxic endpoints of TCDD exposure is suppression of the primary antibody response to SRBC, as determined by the splenic PFC response. In our experiments, CSTM reduced immunosuppression caused by TCDD (Fig. 7). Typically, immunosuppression produced by dioxin involves a mechanism independent of the AhR-mediated pathways as previously reported (Matsumura *et al.*, 1984; Bombick *et al.*, 1985; Beebe *et al.*, 1990; Kerkvliet *et al.*, 1990). However, our data suggest that amelioration of TCDD-induced suppression of the PFC response is associated with AhR.

Our results indicated that the protective effect of CSTM against toxicity of TCDD was based on its ability to suppress activation of AhR and to enhance an antibody-producing response. Another possibility is that CSTM may reduce TCDD toxicity via its effect as an antioxidant and free radical quencher. This possibility remains to be clarified.

Conclusion

We have shown that CSTM reduces adverse effects such as the loss of body weight gain and lethality produced by TCDD. Although the mechanism is not yet fully understood, CSTM seems to exhibit the above effects through different pathways including inhibition of AhR activation and production of immunostimulation. This is the first time a report has demonstrated the possibility of melanin suppressing AhR that may provide new insights into the development of therapeutic and preventive approaches for dioxin toxicity. These results suggest that CSTM may be a promising source for the research and development of potential protection against TCDD-induced pathogenesis.

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