Protective Effects of Tea Melanin against 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin-Induced Toxicity: Antioxidant Activity and Aryl Hydrocarbon Receptor Suppressive Effect

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We examined the protective ability of tea melanin against 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD)-induced toxicity in C57BL6J mice. Reduced tea melanin (RTM) and non-reduced tea melanin (NRTM) were incorporated to distinguish anti-oxidant activity from alternative pathways. The mice were given a single oral dose of TCDD (100 µg/kg body weight) and then they were administered daily with NRTM or RTM (40 mg/kg, *p.o.*) for next 14 d. RTM protected the animals against TCDD-induced lipid peroxidation, inhibition of glutathione peroxidase, alteration in reduced and oxidized glutathione concentrations, loss of body weight, and increased relative liver weight. NRTM was less effective as compared to RTM because of its inferior antioxidant activity, but it still displayed a strong protective effect against TCDD toxicity owing to its similar suppression of the activity of the aryl hydrocarbon receptor. Both NRTM and RTM suppressed the expression of *CYP1A1* gene and prevented the activation of cytochrome P450 isozyme in the livers of animals exposed to TCDD. These results suggest that tea melanin might be a potential agent offering dual protection against the development of TCDD-induced oxidative stress.

Key words tea melanin; antioxidant activity; 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; oxidative stress; aryl hydrocarbon receptor; cytochrome P450

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) is one of the most potent toxicants; it displays a wide spectrum of toxic effects, including dermal toxicity, immunotoxicity, hepatotoxicity, carcinogenicity, teratogenicity, neurobehavioral, endocrine, and metabolic alterations.¹⁾ Recent studies have demonstrated that oxidative stress is an important constituent in the mechanism of TCDD toxicity.^{2,3)} The increased production of reactive oxygen species, lipid peroxidation, and DNA and membrane damage are always associated with TCDD exposure.⁴⁾ Several studies have suggested that TCDD causes the activation of the aryl hydrocarbon receptor (AhR), which is a ligand-activated transcription factor in most cell and tissue types.⁵⁾ The interaction of the transformed AhR complex with a specific dioxin responsive element (DRE) of the nuclear DNA elicits the activation of certain genes, including *CYP1A1*, in the cytochrome P450 superfamily^{6–9)}; this gene is responsible for the development of subsequent toxicity.^{10–12)} In particular, the induction of *CYP1A1* leads to excessive generation of reactive oxygen species as a result of the depletion of cellular antioxidants.^{13,14)}

A number of antioxidants have been tested as possible protectors against TCDD toxicity. Most notably, the protective effects of vitamins A and E have been demonstrated in experiments using TCDD-sensitive C57BL/6J female mice.¹⁵⁾ The natural polyphenol antioxidants have also been recognized as effective defenses against the toxic and carcinogenic effects of TCDD.¹⁶⁾ Polyphenols appear to offer the most protection against TCDD because of their antagonistic interactions with AhR. For instance, antioxidants derived from green tea compete strongly with TCDD for binding to AhR.¹⁷⁾ In addition, polyphenol antioxidants can suppress activation of *CYP1A1* gene.^{18,19)}

Tea melanin displays a wide range of biochemical and

pharmacological activities in animals, including antioxidant, free radical scavenging, and immunomodulatory effects.^{20–22)} Tea melanin also exhibits a protective activity against a number of toxic substances, such as benzidine, hydrazine, and snake venoms.^{23–26)} The current study was undertaken based on the multiple bioactivities of tea melanin. We employed preparations of a reduced form of tea melanin (RTM) and a non-reduced tea melanin (NRTM). Employing both RTM and NRTM allowed us to perform a comparative investigation of the protective effects as a function of their antioxidant activities.

MATERIALS AND METHODS

Materials The tea was manufactured in Miaoli, Taiwan. TCDD was purchased from AccuStandard (New Haven, CT, U.S.A.). [³²P]ATP was purchased from Amersham (Piscataway, NJ, U.S.A.) and dimethyl sulfoxide (DMSO) was obtained from Fisher Scientific (Pittsburgh, PA, U.S.A.). T4 polynucleotide kinase and a Sephadex G-25 spin column were purchased from Roche Diagnostics, Co. (Indianapolis, IN, U.S.A.). Other chemicals and solvents were of analytical or HPLC grade.

Purification and Characterization of Melanin from Tea The isolation of tea melanin was performed as reported previously.²²⁾ Initially, the polyphenols were removed through treatment of the tealeaves with boiling water at a volume ratio of 1 : 10 (solid/liquid) for 10 min, followed by filtration. The solid matter obtained was immersed in water at a temperature 40 °C, at a volume ratio of 1 : 10, and then the pH was adjusted to 10.5 upon the addition of 10% NH₄OH. The extraction was performed over 12 h. The extract was filtered, acidified to pH 2.5 with 2 N HCl, and centrifuged at 15000 *g*

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for 15 min to collect the pellet. Acid hydrolysis of this pellet was employed to remove any residual carbohydrates and proteins.²⁷⁾ Organic solvents (chloroform, ethyl acetate, and ethanol) were used to remove any lipids and related compounds. Finally, the non-reduced tea melanin (NRTM) was obtained. NRTM was reduced upon treatment with TiCl_3 according to the described procedure.²⁸⁾ The reduced tea melanin (RTM) was dialyzed against milli-Q water for 24 h to remove any residual Ti_3^+ . NRTM and RTM were suspended in distilled water and then 0.1 M NaOH was added dropwise to dissolve the samples and adjust the pH to 7.0. The solutions were filtered through a Nalgene 0.45-mm syringe filter. All operations were conducted under an atmosphere of N_2 .

NRTM and RTM were fractionated on a Sephadex G-75 column (1.6×40 cm) eluting with 50 mM phosphate buffer (pH 7.5) at a flow rate of 0.5 ml/min; the fractions were monitored at 280 nm. Ultraviolet-visible (UV-Vis) absorption spectra were recorded on a JASCO V-530 UV-Visible spectrophotometer. Infrared (IR) spectra were recorded on a Perkin-Elmer 1600 FT spectrometer.

The antioxidant activity of tea melanin was evaluated using the oxidation of linoleic acid as a model reaction.²⁹⁾ Briefly, an emulsion (5 ml) containing linoleic acid (0.4 mg/ml), Tween-20 (4 mg/ml), and β -carotene (50 mg/ml) was mixed with NRTM or RTM solution (0.2 ml) at various concentrations. The reaction was performed at 50 °C, in the dark, and under saturation with oxygen. Aliquots (50 μ l) of the reaction mixture were withdrawn at 30-min intervals and the extent of oxidation was determined spectrophotometrically at 470 nm.

Evaluating the Effects of Tea Melanin on AhR Transformation Transformation of AhR was determined through an electrophoretic mobility shift assay using a liver cytosol fraction and a DRE oligonucleotide probe, as described previously.³⁰⁾ A complementary pair of oligonucleotides (5'-GATCTGGCTCTTCTCACGCAACTCCG-3' and 5'-GATCCGGAGTTGCGTGAGAAGAGCCA-3') was synthesized, purified, annealed, and radiolabeled using [^{32}P]-ATP and T4 polynucleotide kinase. The free nucleotides were removed from the labeled DRE probe on a Sephadex G-25 spin column.

The cytosol fraction was prepared from the livers excised from intact C57BL/6J mice using a minor alteration of the described procedure. Namely, liver cytosol was prepared in buffer containing Hepes (25 mM, pH 7.5), EDTA (1 mM), dithiothreitol (1 mM), and glycerol (10% v/v). The cytosolic fraction (4.0 mg/ml protein) was incubated at 20 °C for 10 min in the presence of various concentrations of tea melanin and then incubation was continued for 2 h in the presence of 1 nM TCDD or, in a control experiment, in the absence of TCDD. The ^{32}P -labeled DRE probe (10 fmol) was added and the mixture was incubated for a further 15 min at room temperature. A 10 μ l aliquot of each probe was loaded onto a 4% nonstacking native polyacrylamide gel containing Tris (25 mM), borate (22.5 mM), and EDTA (0.25 mM) and then it was subjected to electrophoresis at 50 V for 90 min. The amount of ^{32}P -labeled DRE was determined using a Molecular Dynamics phosphorimager.

Animal Experiments Eight-week-old female C57BL/6J mice were housed at room temperature (22 ± 2 °C), exposed

to a 12-h light/dark cycle, and fed standard rodent chow and water *ad libitum*. Mice were assigned randomly into six groups of 10, including a control group (which did not receive any treatment), two negative control groups (which received NRTM or RTM alone), a positive control group (which received TCDD alone), and two experimental groups (which received both TCDD and NRTM or TCDD and RTM). TCDD was administered in a single dose (100 $\mu\text{g/kg}$ body weight) through oral gavage using corn oil as the vehicle. Four hours after TCDD treatment, tea melanin (40 mg/kg body weight) was administered through gavage; it was then administered once a day for the duration of the study. The animals treated with TCDD alone were given water as a vehicle. The body weights of all of the mice were measured on the first and last days of the experiment. The animals were killed through cervical dislocation on the 15th day of the experiment. The livers were isolated and perfused with normal saline to wash out the blood. The excised liver samples were frozen in liquid N_2 and stored at -70 °C prior to assay.

Assessment of the Effects of Tea Melanin on Oxidative Stress Lipid peroxidation was determined in the liver homogenates by measuring the formation of thiobarbituric acid-reactive substances (TBARS), as described previously.³¹⁾ A 10% (w/v) tissue homogenate was prepared in Tris/KCl buffer (0.05 M Tris, 1.15% KCl; pH 7.4). The reaction mixture contained the homogenate (0.5 ml), phosphoric acid (1% w/v solution; 3 ml), and 6% thiobarbituric acid (1 ml). Samples were incubated at 95 °C for 1 h, cooled on ice, and then mixed with 1-butanol (4 ml). After vortexing, the samples were centrifuged at 3000 rpm; the supernatant from each sample was collected for analysis. The TBARS concentration was determined spectrophotometrically at 535 nm using 1,1,3,3-tetramethoxypropane as a standard.

The glutathione peroxidase (GSH-Px) activity was assessed in cytosolic preparations of livers according to the procedure as described.³²⁾ The reaction was initiated through the addition of hydrogen peroxide (2.2 mM) as the substrate. The change in the absorbance at 340 nm was measured for 1 min; the activity was expressed in units of millimoles of oxidized NADPH per minute per milligram of protein.

The total glutathione (GSH) content was assayed using an enzymatic recycling procedure.³³⁾ The liver sample (100 mg) was homogenized in 0.2 M phosphate buffer (pH 8.0; 1 ml) and the mixture was centrifuged at 12000 rpm for 30 min. The supernatant (0.5 ml) was mixed with 4% 5-sulfosalicylic acid (0.5 ml), maintained for 5 min at 4 °C, and then centrifuged again at 3000 rpm for 10 min. The supernatant obtained (0.5 ml) was mixed with 0.2 M phosphate buffer (pH 8.0; 2 ml) and 10 mM 5,5'-dithiobis-2-nitrobenzoic acid (10 ml). The absorbance was measured at 412 nm and the concentration of GSH was expressed in units of nanomoles per milligram of protein. Quantification of the oxidized glutathione (GSSG) was accomplished through derivatization of GSH using 2-vinylpyridine.³⁴⁾ The protein content was determined using a bicinchoninic acid protein assay kit (Sigma, St. Louis, MO, U.S.A.); bovine serum albumin was the standard.

Assessing the Effects of Tea Melanin on DNA Damage 8-Hydroxydiguanosine (8-OH-dG) was employed as a marker of DNA damage. Detection of 8-OH-dG was performed using minor modifications of the procedures de-

scribed previously.³⁵⁾ Briefly, the liver was washed and homogenized, using Teflon homogenizer, in 5 mM ice-cold phosphate-buffered saline (pH 7.4) containing 20 mM EDTA. After centrifugation (1000 rpm, 10 min, 4°C), the nuclear fraction was suspended in the same buffer and mixed with proteinase K. After incubation for 4 h at 37°C, DNA was extracted twice with phenol, once with phenol/chloroform, and once with chloroform and then precipitated using ethanol. Samples were dissolved in 10 mM Tris/HCl buffer (supplemented with 1 mM EDTA) and digested for 2 h with RNase A (0.1 mg/ml). Deoxyguanosine (dG) was quantified by monitoring the absorbance at 290 nm; 8-OH-dG was detected through HPLC using an electrochemical detector.

Evaluating the Effects of Tea Melanin on Hepatic CYP1A1 Livers were homogenized in 0.15 M KCl (3 ml) using a Teflon/glass homogenizer. The microsomes were prepared through differential centrifugation.³⁶⁾ The microsomal pellet was resuspended in 100 mM potassium phosphate buffer (pH 7.25). Protein concentrations were determined using a bicinchoninic acid assay kit (Sigma, St. Louis, MO, U.S.A.). A fraction of the microsomal suspension was reserved for Western blot analysis. The hepatic CYP1A1 activity was assayed using ethoxyresorufin-*O*-deethylase (EROD). The reaction mixture contained the microsomal proteins, potassium phosphate (50 mmol/l; pH 7.4), ethoxyresorufin (1.5 μ mol/l), and an NADPH regenerating system [MgCl₂·6H₂O (4 mmol/l), glucose-6-phosphate (5 mmol/l), glucose-6-phosphate dehydrogenase (1 unit/ml), and NADPH (0.5 mmol/l)]. The reaction was initiated through the addition of NADPH and stopped after 10 min of incubation at 37°C upon the addition of 20% trichloroacetic acid (0.25 ml). The activity was determined by measuring the formation of resorufin spectrofluorometrically (Hitachi F-3000, Japan; excitation wavelength: 530 nm; emission wavelength: 585 nm). The results are reported in units of nanomoles per minute per milligram of protein.

To evaluate the expression of the CYP1A1 protein, the microsomal protein (20 μ g) was heated for 5 min in boiling loading buffer (6% SDS, 20% sucrose, 0.25 M dithiothreitol, and 0.1% bromophenol 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, U.S.A.) for 1 h. The membrane was washed and incubated with horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature; the CYP1A1 protein was detected using an ECLTM Western blotting chemiluminescence system (Amersham Pharmacia Biotech, U.K.).

Data Analysis Data are presented as mean $\pm$ standard error of mean. The comparisons between groups were performed through a one-way analysis of variance (ANOVA) using Sigma Stat (Jandel Scientific Company, San Rafael, CA, U.S.A.). The differences between groups were considered statistically significant at $p < 0.05$.

RESULTS

Antioxidant Activity of Tea Melanin The phenol groups contribute to the antioxidant activity of tea melanin. To evaluate the antioxidant power of tea melanin, we used the

Fig. 1. The Effects of RTM and NRTM on Kinetic of Oxidation of Linoleic Acid in Comparison to Control (Oxidation without Tea Melanin)

RTM or NRTM were introduced into reaction media in final concentration of 2 μ g/ml. Filled circles represent control; filled triangles represent RTM; and open circles depict NRTM. The vertical bars represent standard errors of mean (S.E.M.).

oxidation of linoleic acid as a model reaction. The presence of RTM led to a lag phase for the oxidation, whereas NRTM demonstrated no such lag phase (Fig. 1). The differences in these antioxidant effects can be interpreted on the basis of free radical chain reaction mechanisms.³⁷⁾ RTM behaves as a chain-breaking antioxidant that prevents the propagation of free radicals through reactions between the phenol groups and the peroxide radicals. NRTM is not capable of terminating free radical chain reactions because of its lack of phenol groups, although its quinone groups can decrease the oxidation rate slightly²⁵⁾ through their reactions with alkyl free radicals.

RTM and NRTM Displayed Protective Effects against TCDD-Treated C57BL6J Mice Table 1 presents the measured oxidative stresses developed in the mouse livers following acute TCDD exposure. We observed a fourfold increase in the concentration of TBARS—relative to that in control mice—upon treatment with TCDD at 100 μ g/kg. Daily administering RTM or NRTM to the animals who received TCDD inhibited lipid peroxidation in their livers. RTM had a higher inhibitory effect than did NRTM; *i.e.*, treatment with RTM resulted in a 3.4-fold lowering of lipid peroxidation when compared with the situation where TCDD was administered alone, whereas NRTM reduced the degree of lipid peroxidation 1.7-fold prior to TCDD treatment. Thus, NRTM was about half as effective as RTM. RTM or NRTM introduced alone produced small changes in the degree of lipid peroxidation relative to that observed for the control. In particular, the TBARS levels decreased 1.2- and 1.3-fold following treatment with RTM and NRTM, respectively.

The total hepatic glutathione (GSH) level increased in the TCDD-treated group of animals 1.6 times above the level we observed for the control (Table 1). Treating the mice with either NRTM or RTM did not produce significant changes in the GSH level. However, administering RTM or NRTM to animals who had received TCDD restored the GSH level to that of the control.

Administering TCDD increased the GSSG level and the GSSG/GSH ratio by 2.5 and 1.5 times relative to those of the control. The animals treated with either NRTM or RTM did

Table 1. Effects of Tea Melanin on the TCDD-Induced Oxidative Stress in the Livers of C57BL/6J Mice^{a)}

Treatment	TBARS (nmol/mg prot.)	GSH-Px (nmol/min/mg prot.)	GSH (nmol/mg prot.)	GSSG (nmol/mg prot.)	GSSG/GSH ratio
Control	1.61 ± 0.14	102 ± 9.8	30.4 ± 3.3	2.5 ± 0.3	0.083 ± 0.008
TCDD	6.43 ± 0.12*	51 ± 6.1*	47.5 ± 7.2*	6.2 ± 0.5*	0.13 ± 0.016*
RTM	1.32 ± 0.08***	221 ± 20.3***	29.1 ± 3.0**	2.4 ± 0.3**	0.082 ± 0.005**
TCDD+RTM	1.91 ± 0.2**	114 ± 10.2**	28.2 ± 3.0**	2.2 ± 0.2**	0.078 ± 0.07**
NRTM	1.27 ± 0.13***	142 ± 12.5***	31.1 ± 2.5**	2.5 ± 0.4**	0.08 ± 0.008**
TCDD+NRTM	3.79 ± 0.42***	79 ± 6.9***	35 ± 2.8**	3.5 ± 0.5***	0.097 ± 0.005**

a) Data expressed as mean ± S.E.M. * Significantly different from control at $p < 0.05$; ** Significantly different from TCDD treatment at $p < 0.05$.

Fig. 2. The Suppressive Effects of RTM and NRTM on Accumulation 8-OH-dG in Liver Tissue Induced by TCDD

Data expressed as means ± S.E.M. "a" depicts data significantly different from control with $p < 0.05$; "b" depicts data significantly different from TCDD treatment with $p < 0.05$.

not experience significant changes in their GSSG levels, but those treated with both TCDD and NRTM or with both TCDD and RTM had lowered levels of GSSG. In these cases, however, RTM was more effective than NRTM in lowering the amount of GSSG to the control level. NRTM tended toward normalization of the GSSG concentration, but its level remained 1.4 times higher than that in the control group. Treatment with NRTM also reduced the GSSG/GSH ratio to a level that was statistically indistinguishable from that of the control.

TCDD inhibited GSH-Px activity (Table 1). Administering RTM alone caused a 2.2-fold stimulation of the GSH-Px activity relative to that of the control. Treatment of mice with both TCDD and RTM restored the GSH-Px activity to the control level. Administering NRTM alone produced a 1.4-fold increase in the enzyme's activity relative to that of the control. Co-treatment of the animals with TCDD and NRTM reduced the GSH-Px level, but it remained 20% lower than that observed in the control group.

Protection of TCDD-Induced DNA Damage Figure 2 demonstrates the changes in the 8-OH-dG level following administration of TCDD alone and combined with RTM and NRTM. We found that a single dose of TCDD considerably increased the accumulation of 8-OH-dG in the livers of the mice to a level that was 3.4-fold higher than that in the control group. Treatment with RTM or NRTM did not change the level of 8-OH-dG, but the application of either RTM or NRTM to animals treated with TCDD had a significant protective effect, with the former demonstrating a greater protective power than the latter. In particular, RTM was 1.75 times more efficient in suppressing the accumulation of 8-OH-dG than was NRTM (Fig. 2).

EROD Activity We found that TCDD elevated the hepatic EROD activity, whereas the administration of tea

Fig. 3. Inhibitory Effects of RTM and NRTM on EROD Activity in Liver Microsomes Isolated from C57BL/6J Mice

Data represent means ± S.E.M. "a" depicts data significantly different from control with $p < 0.05$; "b" depicts data significantly different from TCDD treatment with $p < 0.05$.

melanin alone (RTM or NRTM) had no apparent effect (Fig. 3). That is to say, the EROD activity increased 16-fold in TCDD-treated mice relative to that found in the control group. Administering RTM daily did not elicit any notable change in hepatic EROD when compared with the control, but treatment with both TCDD and RTM lowered the EROD activity to 50% of the level found in the control. Thus, co-administration of RTM and TCDD significantly inhibited the activation of *CYP1A1* gene. The animals treated with both NRTM and TCDD experienced a lower degree of protection in terms of the inhibition of EROD activity (Fig. 3).

Treatment of the animals with TCDD and tea melanin changed the degree of expression of CYP1A1. The profile of hepatic CYP1A1 protein expression (Fig. 4) is different to the microsomal EROD activity profile (Fig. 3). We observed that the expression of CYP1A1 was very low in the livers of the untreated animals and in those of the RTM or NRTM groups. Administering TCDD (100 µg/kg, *p.o.*) caused a 20-fold increase in protein expression relative to that of the control group. The application of RTM or NRTM in addition to TCDD treatment decreased the expression of CYP1A1 significantly; RTM and NRTM exhibited approximately the same inhibitory effects (Fig. 4).

Effects of RTM and NRTM on Mortality and Body Weight Table 2 summarizes the data we obtained in regard to the mortality and changes in body and liver weight of the experimental animals. TCDD administration decreased the body weight 2.3-fold and increased the liver weight 1.5-fold relative to those values we determined in the control group. Treatment with NRTM alone had no significant effect on these parameters, relative to the control group. Treatment with RTM alone produced a body weight gain that exceeded that of the control by 46%, but it did not alter the liver weight. Administering RTM provided protection against TCDD-induced body weight loss and prevented the develop-

Fig. 4. Expression of CYP1A1 in Liver of C57BL/6J Mice Exposed to TCDD and/or to RTM and NRTM

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. Data presented are means $\pm$ S.E.M. "a" depicts data significantly different from control with $p < 0.05$; "b" depicts data significantly different from TCDD treatment with $p < 0.05$.

Table 2. Effects of NRTM and RTM on the TCDD-Induced Mortality and Weight Changes in C57BL/6J Mice^{a)}

Treatment	Body weight (g)	Liver weight (% of body weight)	Mortality (%)
Control	Gain 2.8 ± 0.3	5.1 ± 0.4	0
TCDD (100 $\mu\text{g/kg}$)	Loss $1.2 \pm 0.1^*$	$7.8 \pm 0.6^*$	40
NRTM (40 mg/kg)	Gain $2.8 \pm 0.3^{**}$	$5.3 \pm 0.366^{**}$	0
TCDD+NRTM (40 mg/kg)	Loss $0.1 \pm 0.02^{***}$	6.8 ± 0.5	20
RTM (40 mg/kg)	Gain $4.1 \pm 0.5^{***}$	$5.5 \pm 0.4^{**}$	0
TCDD+RTM (40 mg/kg)	Gain $2.2 \pm 0.4^{**}$	$5.9 \pm 0.5^{**}$	10

a) Data expressed as mean $\pm$ S.E.M. * Significantly different from control at $p < 0.05$; ** Significantly different from TCDD treatment at $p < 0.05$.

ment of hepatomegaly; these parameters were not significantly different from those of the control. NRTM provided partial protection against TCDD's toxicity, but it was unable to prevent body weight loss, even though the weight loss was 12 times lower than that observed when TCDD was administered alone. In addition, the lower protective effect of NRTM resulted in the liver weight increasing by 1.3 times over the level of the control.

A single dose of 100 $\mu\text{g/kg}$ of TCDD induced an essential toxicity that resulted in 40% mortality of the experimental animals (Table 2). This TCDD-induced mortality decreased to 10% upon administering RTM. NRTM also provided partial protection, but the mortality reached 20% when RTM was added. No mortality occurred when we administered NRTM and RTM alone.

Melanin Suppresses AhR Transformation To determine whether tea melanin can suppress AhR transformation, we incubated the cytosolic fraction—derived from intact C57B/L6 mice and different concentrations of RTM and NRTM—in the presence of 1 nM TCDD for 2 h. In electrophoresis mobility shift assay we have identified band that belongs to AhR-DRE complex (Fig. 5). The other bands apparently are formed by non-specific interaction of cytosol proteins with DRE and by DRE itself. Those bands were independent of the presence of TCDD in the reaction mixture and stayed unchanged when specific antibody to AhR recep-

Fig. 5. Radiogram Representing Suppressive Effects of RTM (Upper Panel) and NRTM (Lower Panel) on Activation of AhR in Liver Cytosol of C57BL/6J Mice

tor was employed while the upper band was eliminated with antibody treatment. When we subjected the resultant mixtures to electrophoretic mobility shift assays we found that both preparations of tea melanin suppressed the AhR transformation induced by 1 nM TCDD in a dose-dependent manner (Fig. 5). We determined the 50% inhibitory concentrations (IC_{50}) from the regression lines representing the percentages of transformed AhR versus the logarithm of the concentration of tea melanin. The calculated values of IC_{50} for RTM and NRTM were 20.4 ± 2 and 19.5 ± 2 $\mu\text{g/mL}$, re-

spectively; these values suggest an insignificant difference in their antagonistic effects against TCDD.

DISCUSSION

Our *in vivo* experiments demonstrate that both preparations of tea melanin displayed protective effects against TCDD, but the extent of protection differed depending on the antioxidant power of the melanin preparation. For example, daily administering RTM or NRTM to animals pretreated with TCDD inhibited lipid peroxidation in the liver, but RTM had a higher inhibitory effect when compared with that of NRTM (Table 1). We observed a correlation between the antioxidant effects of the tea melanin and the glutathione concentrations in the livers of the C57BL6J mice that received NRTM and RTM after exposure to TCDD. The total hepatic glutathione level increased in the TCDD-treated group of animals (Table 1), but administering RTM or NRTM to the animals pretreated with TCDD reduced the GSH level. The protective effect of RTM was higher than that of NRTM, but we observed no significant differences in their effects. Notably, RTM exerted a higher effect on the GSSG level. The increased level of GSSG that we observed in the group of animals treated with TCDD was reduced in the mice that received a combination of TCDD and RTM. NRTM also tended to normalize GSSG, but its effect on the level of oxidized glutathione was less noticeable, most likely because of its weaker antioxidant power.

Previous studies have concluded that TCDD inhibits glutathione peroxidase, the enzyme involved in the reduction of GSSG. GSH-Px is important for the removal of peroxides from cells.^{14,38,39} The increased lipid peroxidation induced by TCDD is associated with the degree of inhibition of GSH-Px activity (Table 1). In our present study, we also have observed inhibition of GSH-Px activity and increased mortality of C57BL6J mice upon administering TCDD. Treatment with RTM or NRTM protected the C57BL6J mice against TCDD, with the degree of protection correlating with the antioxidant activity of the tea melanin.

Although the detailed mechanism through which TCDD causes sustained oxidative stress remains unclear, the participation of CYP1A1 in the TCDD-mediated production of hydrogen peroxide has been demonstrated.⁴⁰ The mechanism involves transfer of two electrons from NADPH to molecular oxygen. It is likely that in the course of this electron transfer, some of the activated oxygen can be released in the form of superoxide radical anions or H₂O₂. Cytochrome P450 has been demonstrated to contribute to the total cellular production of reactive oxygen in the liver.⁴¹ Increased CYP1A1 activity is associated with TCDD-mediated oxidative stress.⁴²

TCDD is known to be responsible for the carcinogenic effects associated with oxidative DNA damage. The increase in 8-oxo-2-deoxyguanosine production is one of the key pieces of evidence for the possible involvement of CYP in oxidative DNA damage.⁴³

We observed that a single dose (100 µg/kg) of TCDD dramatically increased the accumulation of 8-OH-dG in the livers of mice. Treatment with RTM or NRTM alone did not change the level of 8-OH-dG, but administering either RTM or NRTM to animals exposed to TCDD had a significant protective effect, more so for the former than for the latter: the

suppression of accumulation of 8-OH-dG was 1.75 times more efficient in the presence of RTM than in the presence of NRTM (Fig. 2). Thus, the protection of DNA correlates with the antioxidant activity of the tea melanin.

The development of oxidative stress in the livers of mice during TCDD exposure is associated with the activation of *CYP1A1* gene. In our present experiments we found (Fig. 3) that TCDD increased the level of microsomal EROD, which reflects the activation of *CYP1A1*; in fact, the *CYP1A1* activity increased in the animals treated with 100 µg/kg TCDD by 16-fold relative to that of the control. Administering tea melanin significantly inhibited the activation of this isozyme. The profile of the hepatic CYP1A1 protein expression, as determined from a Western blot (Fig. 4), was different from the profile of the microsomal EROD activity (Fig. 3). Administering NRTM or RTM to the animals treated with TCDD decreased *CYP1A1* expression levels evenly, almost to the level we observed in the control. Thus, both NRTM and RTM display similar and strong inhibitory effects on CYP1A1; *i.e.*, these effects are not connected with their antioxidant activity.

TCDD toxicity is known to be associated with the transformation of AhR.^{44–46} We have demonstrated the dose-dependent suppression of AhR (Fig. 5). Previous reports have provided evidence that green tea extract and its major compounds, catechins, suppressed AhR transformation and CYP1A gene expression,⁴⁷ but the effects that we observed to be caused by both RTM and NRTM are not associated with the presence monomeric polyphenols. Careful purification and analysis of our RTM and NRTM samples indicated that these preparations were free from known any biologically active components of black tea, *i.e.* caffeine, catechin, epicatechin, epicatechin gallate, epigallocatechin, epigallocatechin gallate, and theaflavine.

Our evaluation of the antagonistic effects of NRTM and RTM toward AhR provided IC₅₀ values of 19.5 and 20.4 µg/ml, respectively. Green tea extract suppresses AhR transformation to *ca.* 50% at concentrations of *ca.* 250–500 µg/ml.¹⁷ Thus, the effects of NRTM and RTM were both much stronger than that of the green tea extract. This cause of this extraordinary effect of tea melanin remains unclear, but a similar phenomenon was reported previously with respect to the formation of a microsome-catalyzed [3H]-B(a)P-derived DNA adduct in the presence of a polymeric polyphenol from tea.⁴⁸ It was suggested that the polymeric polyphenols in black tea caused relatively higher inhibitory effects when compared with those of the parent monomeric compounds. Likely, this effect is associated with the unique properties of the polymeric matrix of melanin.

In this study we have found that both antagonistic and antioxidant properties of tea melanin are participating in protection against TCDD toxicity. If compare all effects of NRTM alone, *i.e.* compound without essential antioxidant activity (Table 1, Figs. 1–5), one can see that antagonistic effect itself can also able to prevent development of oxidative stress. The mechanism of development oxidative stress is primarily derived from activation AhR receptor involving CYP1A1 in production hydrogen peroxide and super oxide radical. Utilization RTM are strengthen protective effect due to additional antioxidant activity on a top of the antagonistic effects of NRTM. Thus, antioxidant effects of tea melanin are independent of its suppressive effects on activation of

AhR. However, overall protective effect is contributed from both antioxidant and antagonistic effects. Thus, limited efficacy of AhR agonists unable to provide sufficient protection without using of antioxidants. Tea melanin has ability to render dual protection because of combination agonistic and antioxidant properties.

Understanding the antagonistic effects that tea melanins display toward AhR will allow us to exploit their protective activities against TCDD. It is well known that AhR is a ligand-dependent transcription factor that mediates the biological and toxic effects of different chemical species.⁴⁵⁾ Binding ligands to the AhR complex stimulates a conformational change in AhR. In the nucleus, AhR dissociates from its associated protein subunits and dimerization with its nuclear protein partner—the aryl hydrocarbon receptor nuclear translocator (AHRNT)—converts the AhR complex into its DNA-binding form.⁴⁹⁾ 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.⁵⁾ The most responsive genes are those involved in xenobiotic metabolism, e.g., the cytochrome P450 isozymes CYP1A1, CYP1A2, and CYP1B1.

Regarding the antagonistic mechanism of tea melanin on AhR, the molecule of melanin is too large to fit into AhR binding pocket.⁵⁰⁾ Thus, antagonistic effect is more likely due to non-specific interaction. We found that tea melanin can affect AhR transformation when applied either before or after addition TCDD suggesting that melanin inhibit binding of the transformed AhR to DRE probe in addition to the binding of TCDD to AhR. Therefore, tea melanin is non-specific compound, which can reduce AhR transformation even after exposure by inhibiting of transformed AhR to DNA.

In conclusion, we have demonstrated that RTM and NRTM both reduce the adverse effects caused by TCDD, such as the loss of body weight gain and lethality. Both of these melanin preparations exhibited these effects through their inhibition of AhR activation, but RTM demonstrated a higher efficiency because of its superior antioxidant properties. This report is the first to demonstrate the possibility that melanin can suppress AhR; this finding may provide new insight into the development of therapeutic and preventive approaches toward dioxin toxicity. Because of its antioxidant properties, tea melanin can be utilized as a dual protector against the toxicity of TCDD, which is accompanied by an increase in oxidative stress. Our results suggest that tea melanin is a promising source for the development of potential protectors against TCDD-induced oxidative stress and, especially, against DNA damage; thus, it may reduce the risk of carcinogenesis.

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