The Plant Flavonoid, Quercetin, Reduces Some Forms of Dioxin Toxicity by Mechanism Distinct from Aryl Hydrocarbon Receptor Activation, Heat-Shock Protein Induction and Quenching Oxidative Stress

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(Received December 27, 2004; Accepted March 28, 2005)

To clarify the relationship between heat shock protein (Hsp) 70 expression and dioxin toxicity, we studied the effect of quercetin, which is an inhibitor of the biosynthesis of Hsp70, on the acute toxicity of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) in C57BL/6J mice. Contrary to our expectation, results showed that continuous administration of quercetin could attenuate the reduction in body weight gain produced by TCDD (100 μg/kg, *p.o.*). In addition, the hepatomegaly was also significantly reduced by treatment with quercetin for 1 and 30 day(s) after TCDD treatment in spite of having no effect on thymic atrophy. On the other hand, quercetin showed no notable effects on TCDD-induced changes in hepatic *Hsp70.1* mRNA, ethoxyresorufin *O*-deethylase activity and lipid peroxidation. Although we failed to clarify the exact protective mechanisms, our data suggest that quercetin has a protective effect against some forms of dioxin toxicity.

Key words —— quercetin, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, heat shock protein, attenuation, toxicity, wasting syndrome

INTRODUCTION

Some dioxins, like the polychlorinated dibenzo-*p*-dioxins, are widespread, persistent and highly toxic environmental pollutants. Among these dioxins, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) is the most toxic congener and has been widely investigated as a model compound for this class of chemicals. It is well-known that TCDD causes a variety of adverse effects including carcinogenesis, reproductive toxicity, immune dysfunction, hepatotoxicity, teratogenicity, and endocrine changes.[1,2] Wasting syndrome in rodents is one of the sub-acute toxicities produced by dioxins and this has been used as an index of TCDD toxicity. Typical features of this syndrome consist of feed refusal, reduction in body weight gain and exhaustion of energy stores.[3] The mechanisms governing TCDD toxicities, including wasting syndrome, remain incompletely understood, although activation of the aryl hydrocarbon receptor (AhR) is thought to play a crucial role.[1)]

In our previous study, we found that heat shock protein (HSP) 70 is induced in the hepatic cytosol of rats following treatment with 3,3′,4,4′,5-pentachlorobiphenyl (IUPAC No. PCB126).[4] HSPs belong to the chaperon group of proteins ubiquitously expressed both in prokaryotes and eukaryotes. Among the various HSP isoforms, the HSP70 family plays a critical role in the cellular response to acute stress. Taking this and our previous data described above into consideration, it is our hypothesis that HSP70 induction is a defensive response in animals to suppress dioxin toxicity. This is partially supported by our previous observation that geranylgeranylacetone and curcumin, potent inducers of Hsp70, significantly attenuated the reduction in body weight gain and lethality in C57BL/6J mice produced by TCDD.[5,6]

Flavonoids are thought to improve health, and this effect seems to be due at least partially to their antioxidant effects. Diets rich in phenolic flavonoids such as quercetin reduce the risk of chronic degen-

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erative diseases associated with free radicals.[7] Quercetin can scavenge superoxide radicals and hydroxyl radicals, reduce lipid peroxy radicals and inhibit lipid peroxidation.[8] In addition, it was recently reported that quercetin reduces the biosynthesis of Hsps by reducing heat shock factor, which is a transcriptional factor contributing to Hsp expression.[9]

The objective of this study is to investigate the effect of quercetin on TCDD — induced wasting syndrome. To obtain information on the mechanism whereby quercetin exhibits this effect, we also analyzed the effect of this flavonoid on AhR, Hsp and lipid peroxidation. The results obtained suggest that quercetin partially attenuates TCDD toxicity without producing any change in AhR function and Hsp70 expression.

MATERIALS AND METHODS

Reagents —— TCDD (purity > 99%) was obtained from AccuStandard, Inc. (New Haven, CT, U.S.A.). A stock solution (40 μg/ml) was prepared by dissolving TCDD in acetone and this was stored at –20°C until use. Quercetin, NADPH, ethoxyresorufin and thiobarbituric acid were purchased from Sigma (St. Louis, MO, U.S.A.). Deoxyribonuclease (DNase) I (Amplification grade) was purchased from Invitrogen Corp. (Carlsbad, CA, U.S.A.). Ex Taq™ DNA polymerase was purchased from Takara Bio Inc. (Ohtsu, Japan). Resorufin sodium salt and 1,1,3,3-tetraethoxypropane were purchased from Aldrich Chemical Co., Inc. (Milwaukee, WI, U.S.A.) and Wako Pure Chemical Industries, Ltd. (Osaka, Japan), respectively. All other chemicals were of analytical grade and commercially available.

Animals and Treatments —— A TCDD stock solution was diluted with corn oil, and the acetone was then evaporated under nitrogen before administration. Quercetin was dissolved in 0.5% Tween 80 at a concentration of 10 mg/ml. Male C57BL/6J mice (4 weeks old) were purchased from CLEA Japan (Tokyo, Japan), and acclimatized for eight days prior to treatment. Throughout the experiments, mice were allowed access to food and water *ad libitum*. The effect of quercetin on TCDD toxicity was estimated in two separate experiments in which different doses of TCDD were given to mice (Experiment 1 and 2). In both experiments, quercetin was administered by gavage at a dose of 100 mg/kg/10 ml on day 0. The dose of quercetin was selected on the basis of literature information which reported Hsp70 suppression in mice at a dose of 100 mg quercetin/kg.[10] Then, 6 hr after treatment, TCDD was given to mice by gavage once at a dose of 100 μg/kg/5 ml corn oil (Experiment 1) or 10 μg/kg/5 ml (Experiment 2). After the initial treatment on day 0, the same dose of quercetin was administered once a day throughout the experiments. The same volume of each vehicle (prepared solution and/or corn oil) was given to control and quercetin-treated groups. While, in Experiment 2, treatment of mice with quercetin was continued for 30 days, three different schedules were performed in Experiment 1 where the effect of quercetin was examined following treatment for 1, 5 and 30 days. The body weights of all mice were monitored throughout the experiments, and the organ weights of all mice were measured on the day following the last administration.

Analysis of *Hsp70.1* mRNA by Semi-Quantitative Reverse Transcriptional (RT)-Polymerase Chain Reaction (PCR) —— Total RNAs of liver (approximately 20 mg) were extracted using a commercial kit, RNeasy® Mini Kit (QIAGEN, GmbH, Hilden, Germany), according to the manufacturer's instructions. After DNase treatment, reverse transcription was performed using SUPERSCRIPT™ II First-strand Synthesis System for RT-PCR (Invitrogen Corp., Carlsbad, CA, U.S.A.) with Oligo(dT)$_{12–18}$ primer. From our preliminary studies, PCR conditions that guarantee a quantitative amplification during PCR were selected as follows. The reaction mixture (50 μl) consisted of 1 μl cDNA diluted with distilled water, 1.25 units Takara Ex Taq™ DNA polymerase, 4 μl attached dNTP mixture (2.5 mM each), 0.2 μM specific primers (Table 1) and 5 μl attached 10 × Ex Taq™ buffer. *β-Actin* cDNA was also amplified with specific primers (Table 1) as a standard for normalization. The cDNA template was diluted 8-fold or 40-fold for the amplification of *Hsp70* and *β-actin* mRNA, respectively. The reaction was carried out under the following conditions: 2 min at 95°C – (1 min at 95°C – 1 min at 64°C – 1 min at 72°C) × adequate cycles – 20 min at 72°C – hold at 4°C. PCR cycles employed were as follows: 32 (mice treated with quercetin for 1 and 5 days) and 30 (quercetin treatment for 30 days). The cycle numbers for *β-actin* amplification in mice treated with quercetin for 1, 5 and 30 days were 30, 28 and 28, respectively. PCR products were separated by agarose gel (2%) electrophoresis and stained with ethidium bromide. The band intensities of PCR products were estimated using NIH image software (version 1.52, Wayrve Rasband, Bethesda, MD, U.S.A.).

Table 1. Primers for PCR Amplification of *Hsp70.1* and *β-Actin* mRNAs and Product Sizes

Gene (GeneBank No.)	Sequence	Product size (bp)
Hsp70.1 (M35021)		
sense	5′-TAA TGT TGG GAG CAG CAC TGT-3′	325
antisense	5′-AGG GTG GCA GTG TAG ACA TGT A-3′	
β-actin (BC040513)		
sense	5′-CAC CAT GTA CCC AGG CAT TGC-3′	194
antisense	5′-AGG GGC CGG ACT CAT CGT ACT-3′	

Measurement of Ethoxyresorufin-*O*-Deethylase (EROD) Activity —— The hepatic EROD activity was measured by the method of Burke and Mayer[11] with minor modifications. Liver was homogenized at 4°C in 1.15% KCl, and centrifuged at 9000 × *g* for 20 min. The supernatant was stored at –80°C until use. The EROD activity was determined in a reaction mixture containing 20 nM 7-ethoxyresorufin, 2 mM NADPH and a sufficient amount of the 9000 × *g* supernatant in a final volume of 1 ml 0.1 M Tris–HCl (pH 7.8). The reaction mixture was preheated at 37°C for 3 min without NADPH, and incubation was then initiated by adding NADPH. The amount of protein in the 9000 × *g* supernatant added and the incubation time determined from our preliminary studies were as follows: control and quercetin-treated groups, 200 *µ*g protein and 3 min; TCDD- and quercetin + TCDD-treated groups (treatment for 5 days), 25 *µ*g protein and 2 min; TCDD- and quercetin + TCDD-treated groups [treatment for 1 and 30 day(s)], 50 *µ*g protein and 2 min. Incubation was stopped by addition of 1 ml cold acetone, and then the reaction mixture was centrifuged at 4°C and 3000 rpm for 15 min. After centrifugation, 100 *µ*l of the supernatant was transferred to a 96-well plate. The resorufin produced was measured using a Fluoroskan Ascent FL microplate fluorometer (Labsystems, Helsinki, Finland) with the following filters: 544 nm for excitation and 590 nm for emission. The blank control contained no NADPH.

Measurement of the Concentration of Thiobarbituric Acid Reactive Substances (TBARS) —— The amount of hepatic TBARS was measured by the method of Ohkawa *et al.*[12] Livers were homogenized at 4°C in 10 times their volume of 1.15% KCl. The reaction mixture (4 ml) consisted of 0.2 ml 8.1% sodium dodecyl sulfate, 1.5 ml 20% acetic acid (pH 3.5), 1.5 ml 0.8% thiobarbituric acid, 0.1 ml 10% liver homogenate and distilled water. The reaction mixture was heated at 95°C for 60 min, and then the reaction was stopped by addition of 1 ml distilled water and 5 ml *n*-butanol : pyridine mixture. After cooling with water, the reaction mixture was centrifuged at room temperature and 3000 × *g* for 20 min. The absorbance of the organic layer was measured at 532 nm. The calibration curve was constructed by adding 1,1,3,3-tetraethoxypropane as a standard to a solution without liver homogenate.

Other Methods —— The protein content was determined by the method of Lowry *et al.*[13] with bovine serum albumin as a standard. Statistical significance was calculated by Fischer's Protect Least Significant Difference (PLSD) test.

RESULTS

Effect of Quercetin on TCDD-Induced Toxicity

The effect of quercetin on the changes in body weight gain by TCDD is shown in Fig. 1. In Experiment 1 with a high dose of TCDD (100 *µ*g/kg), the body weights increased day by day in mice treated with vehicle or quercetin alone. In contrast, a significant reduction in body weight gain was observed in mice treated with TCDD and quercetin + TCDD, from the beginning at day 4. This reduction in body weight was significantly attenuated in the quercetin + TCDD-treated group in comparison with the TCDD-treated group from day 11. The percentage increase in the body weigh in the TCDD- and quercetin + TCDD-treated groups at day 30 was approximately 1.0 and 9.1%, respectively. In Experiment 2 with a low dose of TCDD (10 *µ*g/kg), the body weights of mice increased similarly in all treatment groups (data not shown). No notable differences in body weight change were observed among control, TCDD- and quercetin + TCDD-treated groups during the experiment. In both Experiments 1 and 2, the food intake by TCDD-treated mice did not differ from that by TCDD-untreated groups (data not shown). Table 2 shows the effects of quercetin and/or TCDD on organ weights. As expected, a signifi-

cant increase in liver weight was observed following TCDD treatment in Experiment 1 and 2. In contrast, this increase was significantly reduced in the quercetin + TCDD-treated group at day 1 and 30 in Experiment 1. Although a significant increase in kidney weight was observed in the TCDD-treated group at day 30 in Experiment 1, this increase was also significantly reduced by co-treatment with quercetin while quercetin had no effect on the change in weights of the other organs produced by TCDD.

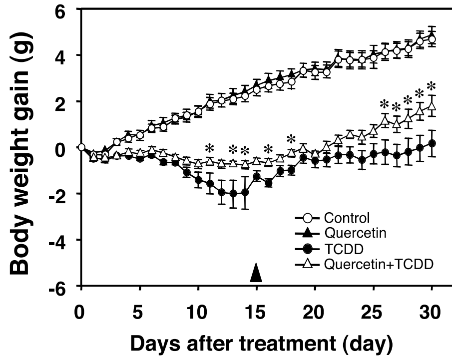

Fig. 1. Effect of Quercetin on Body Weight Gain of C57BL/6J Mice Treated with TCDD

Mice received a single dose of TCDD (100 μg/kg, *p.o.*). The values represent the mean ± S.E. of 9–10 mice. The arrowhead shows the day when a mouse died in the TCDD-treated group. The initial body weights (mean ± S.E.) of mice in control, quercetin-treated, TCDD-treated and quercetin + TCDD-treated mice were 20.0 ± 0.3, 20.1 ± 0.3, 19.9 ± 0.3 and 19.8 ± 0.2 g, respectively. Significant difference between the quercetin + TCDD group and TCDD group; $*p < 0.05$.

Effects of Quercetin on the Hepatic Level of *Hsp70*

To clarify the mechanism governing the effect of quercetin on TCDD toxicity, we examined the levels of hepatic *Hsp70* mRNA in Experiment 1 using semi-quantitative RT-PCR. Among the *Hsp70* isoforms, we focused on *Hsp70.1* because of its higher responsiveness toward acute stimuli compared with the other isoforms. The *Hsp70.1* mRNA expression tended to be increased in the TCDD- and quercetin + TCDD-treated groups in comparison to control at day 1 and 5 (Fig. 2), although the difference was not statistically significant. Co-treatment with quercetin did not produce any effect on *Hsp70.1* expression both in control and TCDD-treated mice. While, at day 30, no notable changes were observed

Table 2. Effects of Quercetin on Organ Weights of C57BL/6J Mice Treated with TCDD

Group	Liver	Thymus	Spleen	Kidney
	(% of body weight)			
Experiment 1				
Day 1				
Control (4)	5.91 ± 0.11	0.26 ± 0.00	0.29 ± 0.02	1.30 ± 0.03
Que (4)	6.00 ± 0.14	0.25 ± 0.02	0.27 ± 0.01	1.31 ± 0.01
TCDD (4)	6.37 ± 0.06**	0.23 ± 0.02	0.26 ± 0.02	1.33 ± 0.02
Que + TCDD (4)	6.02 ± 0.07†	0.24 ± 0.01	0.25 ± 0.01	1.35 ± 0.02
Day 5				
Control (4)	5.80 ± 0.11	0.22 ± 0.02	0.27 ± 0.01	1.35 ± 0.03
Que (4)	5.96 ± 0.18	0.24 ± 0.01	0.28 ± 0.01	1.36 ± 0.03
TCDD (4)	7.48 ± 0.06***	0.09 ± 0.01***	0.26 ± 0.02	1.41 ± 0.04
Que + TCDD (4)	7.85 ± 0.19***	0.10 ± 0.01	0.28 ± 0.01	1.39 ± 0.03
Day 30				
Control (4)	5.20 ± 0.04	0.16 ± 0.01	0.24 ± 0.01	1.29 ± 0.02
Que (4)	5.05 ± 0.05	0.17 ± 0.01	0.25 ± 0.01	1.25 ± 0.02
TCDD (4)	7.89 ± 0.33***	0.07 ± 0.01***	0.26 ± 0.01	1.38 ± 0.01*
Que + TCDD (4)	6.87 ± 0.27***,†	0.07 ± 0.01***	0.26 ± 0.01	1.32 ± 0.01
Experiment 2				
Day 30				
Control (5)	5.09 ± 0.07	0.18 ± 0.01	0.25 ± 0.01	1.31 ± 0.01
TCDD (5)	6.03 ± 0.09***	0.12 ± 0.00***	0.26 ± 0.00	1.31 ± 0.03
Que + TCDD (5)	6.15 ± 0.09***	0.12 ± 0.01***	0.26 ± 0.01	1.25 ± 0.03

The values represent the mean ± S.E. The number of mice is shown in parenthesis. Significant difference compared with control: $*p < 0.05$; $**p < 0.01$; $***p < 0.001$. Significant difference compared with TCDD: $†p < 0.05$. Quercetin is abbreviated as Que in this table.

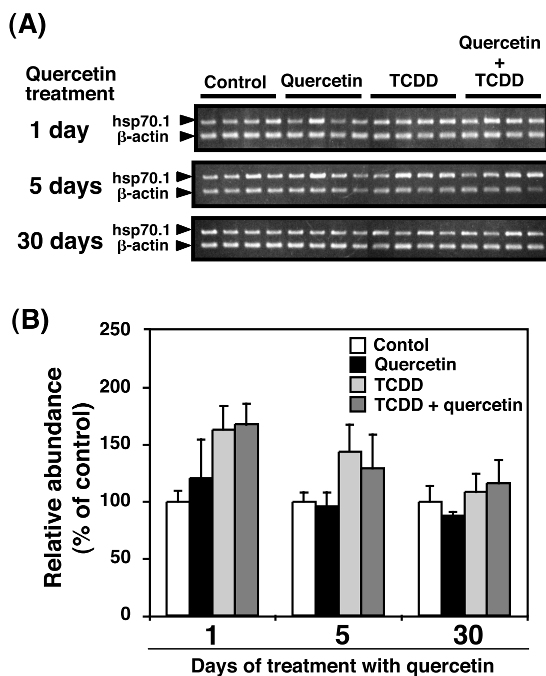

Fig. 2. Effects of Quercetin on *Hsp70.1* mRNA Expression in Liver of C57BL/6J Mice Treated with TCDD

The effect of quercetin was estimated in separate experiments with different periods of treatment (1, 5 and 30 days), following single oral administration of TCDD (100 μg/kg). Band image of amplified cDNA (A) and relative expression to the control (= 100%) (B) are shown. The band intensity of *Hsp70.1* mRNA was normalized by *β-actin* used as a standard mRNA, prior to the comparative analysis.

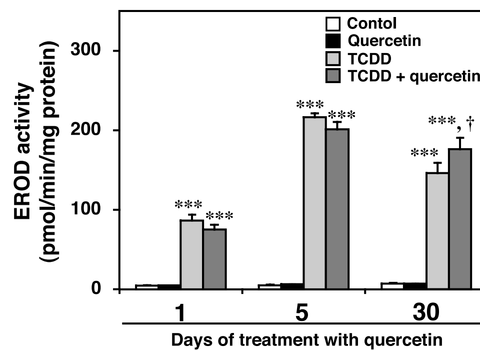

Fig. 3. Effects of quercetin on hepatic EROD activity of C57BL/6J mice treated with TCDD (100 μg/kg, *p.o.*)

Each value represents the mean ± S.E. of 4 or 5 mice. Significant difference compared with each control: $^{***}p < 0.001$; compared with TCDD-treated group: $^{\dagger}p < 0.05$.

among all treatment groups.

Effects of Quercetin on AhR-Mediated Gene Expression

It is widely accepted that dioxins exert their toxicity by altering gene expression through AhR activation. Cytochrome P450 (Cyp) 1a1 is one of the well-known enzymes induced by TCDD-mediated activation of AhR.[14] While quercetin exhibits an antagonist action, such as inhibition of the binding of AhR to xenobiotic responsive element at low concentrations, this flavonoid has a weak agonist effect on AhR at high concentrations.[15] Thus, it is conceivable that quercetin acts by affecting TCDD-mediated activation of AhR-containing pathways. To clarify this possibility, we examined the effect of quercetin on hepatic microsomal EROD activity, which is catalyzed by Cyp1a1. In Experiment 1, the EROD activity was significantly increased by treatment with quercetin + TCDD as well as TCDD alone (Fig. 3). Although long-term treatment of quercetin (30 days) together with initial treatment with TCDD significantly enhanced EROD activity compared with the level obtained by TCDD alone, the increase seemed to be too small to affect TCDD toxicity. Therefore, this result does not support the hypothesis that quercetin reduces TCDD-induced toxicity in mice by interfering with AhR activation.

Effects of Quercetin on Oxidative Stress Produced by TCDD

It is well-established that TCDD induces oxidative stress in animals and causes damage including hepatic lipid peroxidation.[16] Since quercetin is known to exhibit an antioxidant effect,[8] it is possible that quercetin reduces TCDD toxicity by its action on oxidative stresses. To examine this possibility, the amount of hepatic TBARS was measured. As expected, the TBARS concentrations in Experiment 1 were significantly increased in TCDD-treated mice compared with controls (Fig. 4). In contrast, although hepatic TBRAS tended to be lower in quercetin + TCDD-treated mice than in TCDD-treated mice, the difference was not significant. Thus, this result does not support the possibility that quercetin reduces TCDD toxicity by suppressing damage due to oxidative stresses.

DISCUSSION

In the present study, we showed that the TCDD-induced reduction in body weight gain was significantly attenuated by quercetin. Our results also showed that quercetin produces the above effect without affecting Hsp70 expression, activation of AhR and the occurrence of oxidative stress produced

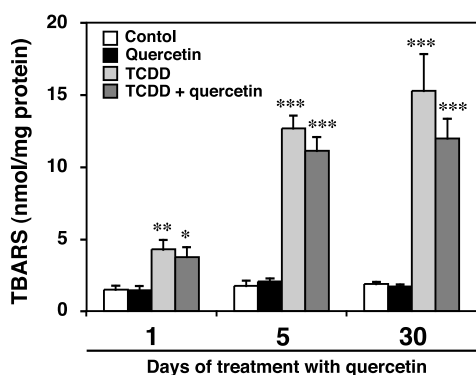

Fig. 4. Effects of Quercetin on Hepatic Lipid Peroxidation of C57BL/6J Mice Treated with TCDD (100 μg/kg, *p.o.*)

TBARS is used as the index of lipid peroxidation. Each value represents the mean ± S.E. of 4 [treatment groups for 1 and 5 day(s)] or 7 (treatment group for 30 days) mice. Significant difference compared with each control: $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

by TCDD (Figs. 2–4). A close relationship between TCDD toxicity and the activation of AhR has been suggested in numerous studies carried out to date. Indeed, AhR-deficient mice are reported to resist the toxic effect of TCDD, including hepatomegaly, thymic atrophy and teratogenicity.[17,18)] Wasting syndrome, including a reduction in body weight gain, is also suggested to occur by an AhR-dependent mechanism.[19)] However, the factors contributing to this syndrome induced by TCDD remain largely unknown. On the other hand, some effects of TCDD, such as immunosuppression, have been shown not to require AhR.[20)] In this report, we were unable to demonstrate any agonist and antagonist effects of quercetin in the activation of AhR by TCDD. Therefore, if quercetin has an ability to attenuate some forms of TCDD toxicity as shown in this report, it would be due to the effect distinct from direct suppression of TCDD-induced AhR activation. The observation that quercetin fails to activate AhR activation in the assay system using luciferase reporter gene supports above view.[21)]

One of our working hypotheses was that the induction of Hsp70 causes an attenuation in dioxin toxicity. Therefore, at the beginning of this study, we expected that the toxicity of TCDD would be enhanced by co-treatment with quercetin, because this substance has been reported to be a suppressor of Hsp biosynthesis.[9)] However, our data showed that the TCDD-induced reduction of body weight was significantly attenuated by quercetin (Fig. 1). In addition, our results also showed that quercetin failed to significantly increase *Hsp70.1* mRNA over the level achieved with TCDD alone (Fig. 2). Therefore, the alteration in hepatic Hsp70.1 expression would not contribute to any protective effect of quercetin on the acute toxic effect of TCDD. The reason why TCDD failed to increase hepatic cytosolic *Hsp70.1* mRNA in this study is unclear. In general, while the expression of Hsps is very low under normal physiological conditions,[22)] those proteins are induced by various stresses such as stimuli by xenochemicals.[23)] Although we previously reported the induction of hepatic Hsp70 by dioxins,[4,5)] one of our previous studies performed with the same dose and schedule of TCDD administration as the present study also failed to show any notable TCDD-dependent increase in *Hsp70.1* mRNA.[6)] It is, therefore, likely that co-presence of unknown factor(s) as well as dioxins is needed for Hsp70 induction.

Although we also examined the effect of quercetin on the activation of AhR and the occurrence of oxidative stress produced by TCDD, no notable differences were observed (Figs. 3 and 4). It has been reported that TCDD accumulates in liver and adipocytes when animals are exposed to it.[24)] Thus, the increased lipid peroxidation in hepatocytes is likely to be due to continuous production of active oxygen species by accumulated TCDD in this organ. In fact, our results showed that the lipid peroxidation in liver increases according to the length of exposure to TCDD (Fig. 4). An early work on the metabolic fate of quercetin reported that only 20% of ingested quercetin is absorbed from the gastrointestinal tract.[25)] Absorbed quercetin or its primary methylated metabolite is metabolized by glucuronidation or sulfation, followed by excretion *via* the bile or urine.[26,27)] It is, therefore, conceivable that the weak attenuation produced by quercetin in this study was due to its short half-life in the body or low accumulation of the activated form at the damage site.

It has been suggested that an increase in tyrosine kinase and protein kinase C activities is involved in the mechanism of TCDD toxicity.[28,29)] As far as this is concerned, the phosphorylation of epidermal growth factor receptor, which is a possible regulator of cell differentiation, cell proliferation and apoptosis, is activated by TCDD.[30)] On the other hand, quercetin is able to inhibit tyrosine kinase and protein kinase C activities by binding to the ATP-binding site of these kinases.[31,32)] Thus, it is possible that the inhibition of TCDD-produced protein phosphorylation is involved in the mechanism by which quercetin reduces TCDD toxicity. This assumption is supported by the following evidence: a part of the

toxicity of TCDD, such as lethality, wasting syndrome and thymic atrophy, is attenuated in c-Src deficient mouse.[33] In addition, quercetin is also reported to inhibit the activity of other kinases, such as phosphatidylinositol-3-kinase[34] and casein kinase.[35] Therefore, it may be conceivable that integrated anti-kinase effects by quercetin contribute to the attenuation of TCDD toxicity.

In conclusion, we have shown that quercetin attenuates the sub-acute adverse effects produced by TCDD. Although the mechanism(s) for this remain to be clarified, quercetin seems to exhibit the above effects without affecting the activation of AhR, Hsp70 expression and oxidative stress. Our results offer new insights into the development of a protective method for combating dioxin toxicity using dietary flavonoids.

Acknowledgements This work was supported in part by a grant, Research on Food and Chemical Safety (Research No. 13150201), from the Ministry of Health and Welfare, Japan.

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