Curcumin Anticipates the Suppressed Body Weight Gain with 2,3,7,8-Tetrachlorodibenzo-*p*-Dioxin in Mice

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The protective effect of curcumin, a potent inducer of heat shock protein (Hsp) 70, against the acute toxicity produced by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) was studied *in vivo* in C57BL/6J mice. Curcumin reduced the loss of body weight gain produced by TCDD regardless of having no effect on hepatomegaly and thymic atrophy. *Hsp70.1* mRNA levels in liver and intestine were unaffected or tended to be reduced by TCDD and/or curcumin treatment. Curcumin also had no effect on the induction of hepatic ethoxyresorufin *O*-deethylase activity by TCDD. These data suggest that curcumin exhibits a protective effect against some forms of dioxin toxicity by a mechanism(s) distinct from the increase in hepatic and intestinal Hsp70 and inhibition of arylhydrocarbon receptor activation.

Key words —— curcumin, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, reduction, toxicity, wasting syndrome

INTRODUCTION

Polychlorinated dibenzo-*p*-dioxins (PCDDs) are widespread, persistent and highly toxic environmental pollutants. Among PCDDs, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) is the most toxic congener and it has been widely investigated as a model compound of this class of chemicals. TCDD is formed by incineration of waste materials and in the procedures involved in herbicide production, paper processing and plastics manufacturing, in particular the production polyvinyl chloride.[1,2] Thus, humans are involuntarily exposed to TCDD through the diet and gradually accumulate it throughout their lifetime. Exposure to TCDD can result in a variety of adverse responses including carcinogenesis, reproductive toxicity, immune dysfunction, hepatotoxicity, tetatogenicity, and endocrine changes.[3–5] Wasting syndrome and lethality in rodent have been used as indices of TCDD toxicity. Typical features of TCDD-induced wasting syndrome are feed refusal, reduction in body weight gain and exhaustion of energy stores.[6] Although numerous efforts have been made to resolve the key lesions and the mechanisms governing wasting syndrome, it remains incompletely understood.

In a previous study, we found that geranylgeranylacetone (GGA), which is used in clinical situations as an antiulcer drug, has the ability to reduce the loss of body weight gain and lethality produced by TCDD (unpublished data). Although the governing mechanisms are unclear, one possibility is that GGA rescues mice from TCDD toxicity by inducing heat shock protein (HSP) 70. HSPs are members of the chaperon group of proteins ubiquitously expressed both in prokaryotes and eukaryotes. While the expression of these proteins is very low under normal physiological conditions,[7] they are induced by various stresses such as stimuli by endocrine factors, xenochemicals and infection.[8] Among the various HSP isoforms, the HSP70 family plays a critical role in the cellular response to acute stresses. We also found that HSP70 is induced in the hepatic cytosol of rats treated with one of the dioxins, 3,3′,4,4′,5-pentachlorobiphenyl (IUPAC No. PCB126).[9] Based on this, we formed the hypothesis that HSP70 induction is a defensive re-

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sponse in animals to suppress dioxin toxicity. This is partially supported by the observation that GGA, a potent inducer of HSP70, reduces dioxin toxicity.

Curcumin (diferuloylmethane, Fig. 1) is a common dietary pigments and a spice contained in curry. Animal experiments have shown that curcumin also acts as an anti-inflammatory, antioxidant, hypocholesterolemic, and antiinfection agent.[10,11] Curcumin has also been shown to exert antiproliferative and anticarcinogenic effects in cell lines and laboratory animals.[12,13] Although many biological functions of curcumin have been identified, the molecular mechanisms underlying its actions remain largely unknown. Sood *et al.*[14] have recently reported that curcumin enhances the expression of HSP70 in HK-2 cells. They also showed that curcumin inhibits Shiga toxin-induced apoptosis and necrosis in HK-2 cells. Thus, it is conceivable that curcumin exerts a cytoprotective action by inducing HSP70.

The objective of this study is to investigate if curcumin can reduce the toxic effects of TCDD in C57BL/6J male mice. The results obtained suggest that simultaneous treatment with curcumin and TCDD partially reduces the loss of body weight by TCDD without any functional change in arylhydrocarbon receptor (AhR).

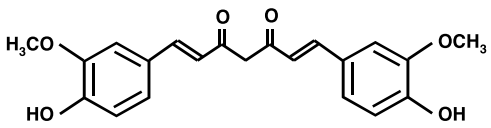

Fig. 1. Structure of Curcumin

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. Curcumin was purchased from Sigma (St. Louis, MO, U.S.A.). Deoxyribonuclease (DNase) I (Amplification grade) and Ex Taq™ DNA polymerase was purchased from Invitrogen Corp. (Carlsbad, CA, U.S.A.) and Takara Bio Inc. (Ohtsu, Japan), respectively. All other chemicals were of analytical grade and commercially available.

Animals and Treatments —— A TCDD stock solution dissolved in acetone was diluted with corn oil, and the acetone was then evaporated under nitrogen before administration. Curcumin was emulsified in a solution containing 5% gum arabic and 0.5% Tween 80. 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 to access to food and water *ad libitum*. The schedule of administration is shown in Fig. 2. On day 0, curcumin was administered by gavage at a dose of 100 mg/kg/5 ml emulsified solution in both experiments. Then, 2 hr after curcumin treatment, TCDD was given by gavage once at a dose of 200 μg/kg/

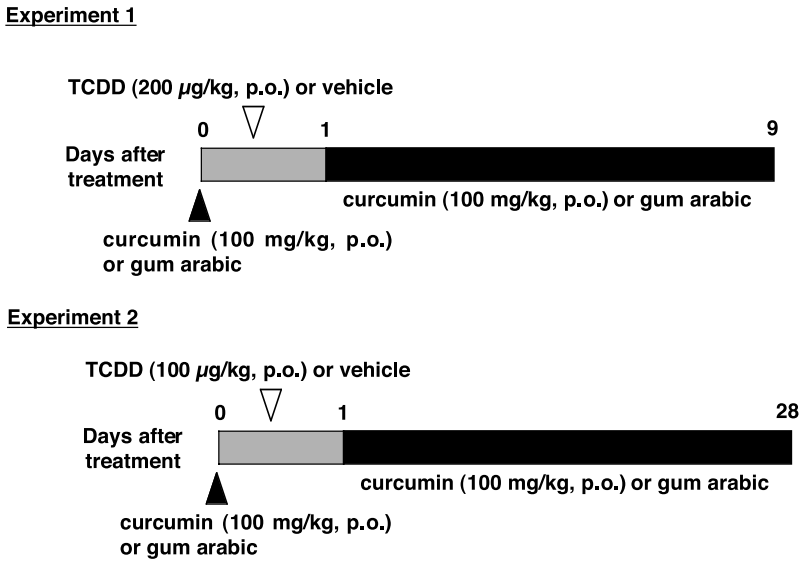

Fig. 2. Administration Schedule of Curcumin and TCDD in Experiment 1 and Experiment 2

Table 1. Primers and Product Sizes

Gene		Sequence	Product size (bp)
Hsp70.1	sense	5′-TAA TGT TGG GAG CAC TGT -3′	325
	antisense	5′-AGG GTG GCA GTG TAG ACA TGT A -3′	
β-actin	sense	5′-CAC CAT GTA CCC AGG CAT TGC -3′	194
	antisense	5′-AGG GGC CGG ACT CAT CGT ACT -3′	

5 ml corn oil (Experiment 1) or 100 μg/kg/5 ml (Experiment 2). After the initial treatment on day 0, the same dose of curcumin was administered once a day throughout the experiments. The same volume of each vehicle (emulsified solution and/or corn oil) was given to mice in the control and curcumin-treated groups. During the study, the body weight of all mice was measured before administration. The organ weights of all mice were measured one day after the last treatment with curcumin.

Analysis of *Hsp70.1* mRNA by Semi-Quantitative Reverse Transcriptional (RT)-Polymerase Chain Reaction (PCR) —— Total RNAs of liver and intestine 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 RNaseH$^-$ Reverse Transcriptase (Invitrogen Corp.) with Oligo(dT)$_{12\text{-}18}$ primer. To ensure quantitative amplification during PCR, the amplifications were carried out under different template content and cycle number conditions. Based on this experiment, PCR conditions that guarantee a dose (template amount)-response relationship were set as follows. The reaction mixture (50 μl) consisted of 0.5 μl cDNA diluted with distilled water, 1.25 units Takara Ex Taq™ DNA polymerase, 0.2 pM specific primers for mouse *Hsp70.1* (Table 1) and 5 μl attached 10 × Ex Taq™ buffer. *β-Actin* cDNA, a standard for normalization, was also amplified using the specific primers (Table 1). The reaction was carried out under the following conditions: 2 min at 94°C – (45 sec at 94°C – 45 sec at 60°C – 75 sec at 72°C) × adequate cycles – 10 min at 72°C – hold at 4°C. Table 2 shows the dilution ratio of cDNA solution and PCR cycles employed. PCR products were separated by agarose gel (2%) electrophoresis and stained with ethidium bromide. The band intensity of PCR products was calculated using NIH image software (version 1.52, Wayrve Rasband, Bethesda, MD, U.S.A.).

Table 2. The Dilution Ratio of cDNA Solution and PCR Cycles Used

organ		cDNA dilution (times)	PCR cycles
Liver	*Hsp70.1*	40	32
	β-actin	40	30
Intestine	*Hsp70.1*	2	33
	β-actin	40	29

Measurement of Ethoxyresorufin-*O*-Deethylase (EROD) Activity —— The hepatic ethoxyresorufin-*O*-deethylase (EROD) activity exhibited by curcumin- and/or TCDD-treated mice was measured by the method of Burke and Mayer[15] with minor modifications. Livers were homogenized at 4°C in 10 mM Tris–HCl (pH 7.5) containing 0.25 M sucrose, 0.1 mM ethylenediamine tetraacetate and 1 mM phenylmethylsulfonyl fluoride, and centrifuged at 9000 × *g* for 20 min. The supernatant obtained 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 100 μg (control and curcumin-treated liver) or 20 μg (TCDD- and curcumin + TCDD-treated liver) protein 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. Incubation was stopped by adding 1 ml cold acetone, then the reaction mixture was centrifuged at 900 × *g* for 10 min. After centrifugation, 100 μl of the supernatant was transferred to a 96-well plate. Production of resorufin 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.

Other Methods —— The protein content of the 9000 × *g* supernatant was determined by the method of Lowry *et al.*[16] with bovine serum albumin as a standard. Statistical significance was calculated by

Fischer's protected least significant difference test.

RESULTS

Effects of Curcumin on TCDD-Induced Toxicity

Prior to the investigation of the effect of curcumin on TCDD toxicity, we examined the acute toxicity of curcumin itself. While higher doses (300 and 900 mg/kg, p.o.) of curcumin tended to reduce the body weights of C57BL/6J mice during a 5 day period following a single administration, lower doses (20 and 100 mg/kg) did not cause such toxicity (data not shown). None of the doses of curcumin had any effect on liver weight. Based on above observation, we employed a dose of 100 mg/kg which was expected to be the maximal dose that would avoid subjecting the mice to stress. The effect of curcumin on the changes in body weight gain by TCDD is shown in Fig. 3. In Experiment 1, the body weight increased day by day in mice treated with vehicle or curcumin alone (Fig. 3A). In contrast, a reduction in body weight gain was observed in mice treated with TCDD and curcumin + TCDD. However, since no notable difference in the rate of body weight change was observed between the TCDD- and curcumin + TCDD-treated groups during the experiment, this study was stopped on day 10. In both groups, the means of the percentage reduction in the TCDD- and curcumin + TCDD-treated groups at day 10 were approximately 6.7% and 5.1%, respectively. In Experiment 2, the body weight was also reduced by treating mice with TCDD and curcumin + TCDD (Fig. 3B). The body weight normally increased in mice treated with vehicle or curcumin alone. In contrast, a reduction in body weight gain was observed in the TCDD- and curcumin + TCDD-treated groups from day 6. Interestingly, the reduction in body weight gain tended to be less in the curcumin + TCDD-treated group in comparison with the TCDD-treated group from day 12. Significant differences in body weight between the two groups (TCDD *vs.* curcumin + TCDD) were detected at many day points after day 21. Table 3 shows the effects of TCDD and/or curcumin on organ weights. As expected, a significant increase in liver weight and atrophy of the thymus were observed following TCDD treatment in both Experiments 1 and 2. However, curcumin had no effect on the TCDD-induced changes in organ weights.

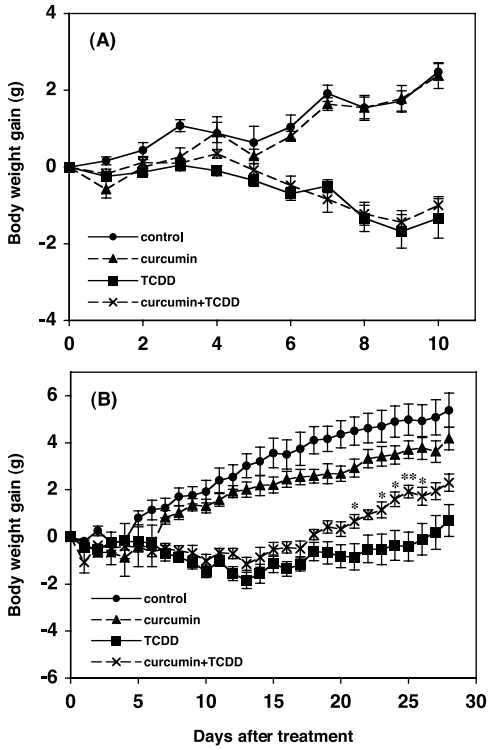

Fig. 3. Effects of Curcumin and TCDD on Body Weight Gain in C57BL/6J Mice

The values represent the mean ± S.E. of 4–7 mice. Before treatment, mice (20 ± 0.13 g) were randomly housed in cage and assigned to treatment groups. Significant difference between the curcumin + TCDD group and the TCDD group; $*p < 0.05$.

Effects of Curcumin on the Hepatic and Intestinal Level of *Hsp70*

To obtain information about the mechanism governing the action of curcumin on TCDD toxicity, we analyzed the hepatic and intestinal levels of *Hsp70* mRNA using semi-quantitative RT-PCR (Fig. 4). Among the *Hsp70* isoforms, we focused on *Hsp70.1* because of its higher responsiveness toward acute stimuli compared with the other isoforms. In both organs, *Hsp70.1* mRNA expression was somewhat reduced in the TCDD- and curcumin + TCDD-treated groups although the difference was not statistically significant. In addition, no significant differences between the TCDD- and curcumin + TCDD-treated groups were detected in the liver and intestine.

Effects of Curcumin on AhR-Mediated Gene Expression

It is widely accepted that dioxins exert their toxicity by altering gene expression through AhR activation. Therefore, it is conceivable that curcumin acts by affecting TCDD-mediated activation of AhR-

Table 3. Change in Organ Weights of C57BL/6J Mice Following Treatment with Curcumin and TCDD

Treatment	Liver	Thymus	Spleen
	(% of body weight)		
Experiment 1			
Control (5)	5.73 ± 0.13	N.D.	N.D.
Curcumin (5)	5.94 ± 0.09	N.D.	N.D.
TCDD (5)	7.09 ± 0.21*	N.D.	N.D.
Curcumin + TCDD (5)	7.18 ± 0.06*	N.D.	N.D.
Experiment 2			
Control (5)	5.40 ± 0.11	0.16 ± 0.02	0.28 ± 0.01
Curcumin (4)	5.45 ± 0.12	0.16 ± 0.01	0.27 ± 0.01
TCDD (7)	8.14 ± 0.24*	0.04 ± 0.01*	0.32 ± 0.04
Curcumin + TCDD (5)	7.28 ± 0.28*	0.06 ± 0.00*	0.28 ± 0.02

The values represent the mean ± S.E. of 4–7 mice. The number of the samples is shown in parenthesis. Significant difference from control: $*p < 0.001$. N.D.: no data.

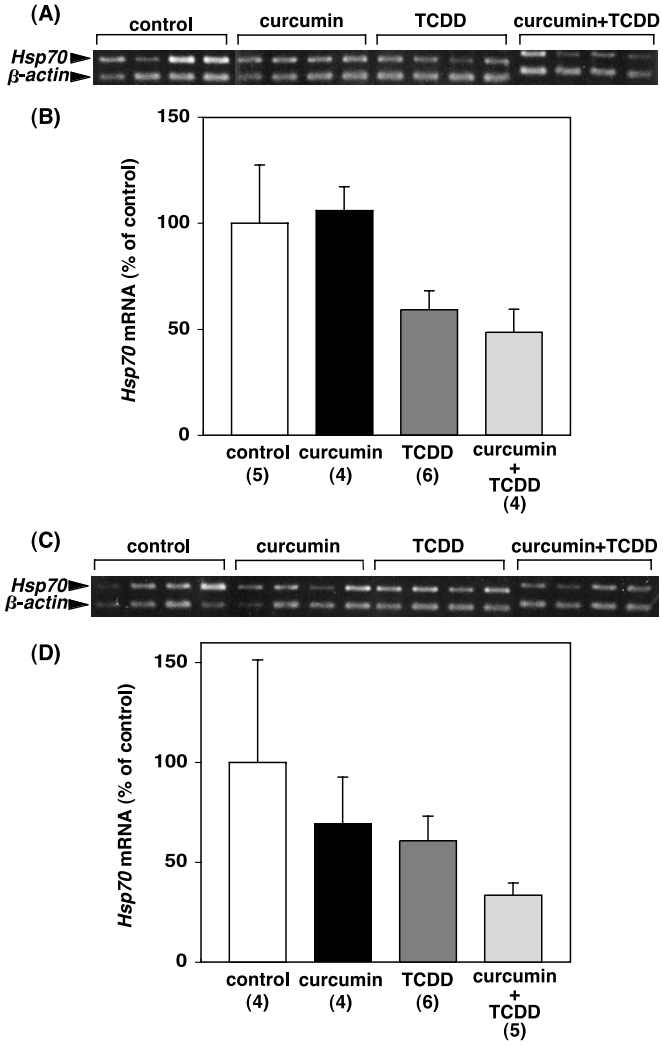

Fig. 4. Analysis of Hepatic (A and B) and Intestinal (C and D) *Hsp70.1* mRNA Expression by Semi-Quantitative RT-PCR in Experiment 2

Total RNA was extracted from the liver and intestine of mice treated with curcumin and TCDD, and the expression of *Hsp70.1* mRNA was investigated using semi-quantitative RT-PCR. The number of samples is shown in parenthesis. The values represent the mean ± S.E. of 4–6 mice. Expression of *Hsp70.1* mRNA (A and C) and relative abundance (% of control, B and C) of *Hsp70.1* mRNA normalized by *β-actin* mRNA are shown.

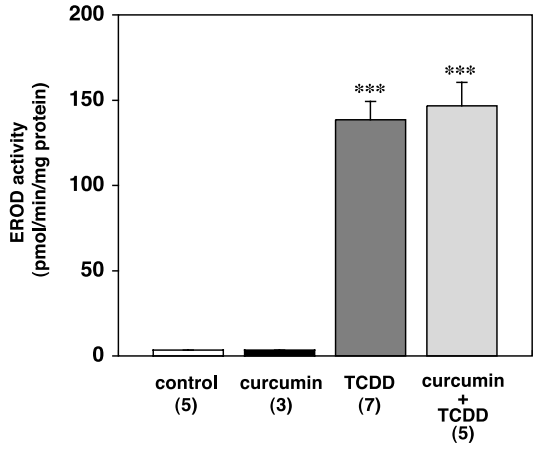

Fig. 5. EROD Activity of Liver Microsomes from C57BL/6J Mice after Exposure to Curcumin and TCDD

The number of samples is shown in parenthesis. The EROD activity of the control was 3.41 ± 0.13 pmol resorufin formed/min/mg protein. The values represent the mean ± S.E. Significant difference from control: ***$p < 0.001$.

containing pathways. To clarify this issue, we next measured the effect of curcumin on hepatic microsomal EROD activity. This activity is catalyzed by cytochrome P450 (Cyp) 1a1, which is one of the well-known enzymes induced by TCDD-mediated activation of AhR.[15] When the 9000 × *g* supernatant of mouse liver in Experiment 2 was used as the enzyme source, the EROD activity was significantly increased by treatment with curcumin + TCDD as well as TCDD alone (Fig. 5). However, no significant difference was found between the TCDD and curcumin + TCDD groups. This result does not support the idea that curcumin reduces TCDD-induced toxicity in mice by interfering with AhR activation.

DISCUSSION

In the present study, our results showed that the TCDD-induced reduction in body weight gain is suppressed or tends to be reduced by pretreatment with curcumin. Our results also suggest that curcumin produces the above effect without affecting the function of AhR. Many studies have demonstrated a close relationship between TCDD toxicity and the activation of AhR. Indeed, AhR-deficient mice are reported to be resistant to the toxic effects of TCDD including hepatomegaly, thymic atrophy and teratogenicity.[17,18] Wasting syndrome, including the reduction in body weight gain, is also expected to be produced through an AhR-dependent mechanism.[19] Some candidate AhR-governed proteins participating in TCDD toxicity have been suggested. These include protein tyrosine kinase c-Src,[20,21] phosphoenolpyruvate carboxykinase,[21] pituitary factors such as adrenocorticotropic hormone,[22,23] β-endorphine-like peptide[24] and proopiomelanocortin.[25] However, the contribution of these factors to the toxic effects of TCDD is largely unknown. On the other hand, some effects of TCDD have been shown not to require AhR. For example, TCDD can produce immunosuppression,[26] induction of protein kinases[27] and phospholipase C,[28] and effects on plasma membranes and low-density lipoprotein receptor *via* AhR-independent mechanisms.[29,30] Although this study failed to observe any significant effect of curucumin on AhR-mediated gene expression, this substance has been reported to exhibit inhibitory effects on various signaling proteins including cyclooxygenase, ornithine decarboxylase, nitric oxide synthase, transcription factors, matrix metalloproteinases, and protein kinases.[31,32] Therefore, it may that curcumin exerts its protective effects by acting on a target(s), such as signal transduction located downstream of the AhR-dependent or -independent pathways.

The hypothesis that the induction of Hsp70 may cause a reduction in dioxin toxicity prompted us to carry out this study. The HSP70 family plays a defensive role against acute stimulation in eukaryotic cells, and the exposure of cells to a variety of stresses activates a survival response *via* induction of the intronless Hsp70.1 and Hsp70.3 genes.[33] Among the members of the HSP70 family, since HSP70.1/70.3 are rapidly expressed in cells in response to acute stress stimuli, these chaperons are considered to be important as quenchers of acute cell damage. Thus, we focused on the alteration of HSP70.1 and examined whether this protein is able to modify TCDD toxicity. Since curcumin failed to significantly increase *Hsp70.1* over the level achieved with TCDD alone (Figs. 4B and 4D), any protective effect of curcumin on an acute toxic effect would not be attributable to altered hepatic and intestinal Hsp70.1 expression. If curcumin exerts a protective effect through Hsp70 induction, it would occur in organs other than liver and intestine. The reason that TCDD did not increase hepatic cytosolic *Hsp70.1* mRNA in this study is unclear. Although the data are not shown, we performed a series of toxicity studies in which the dose and schedule of TCDD administration were the same as in the present study. In some of those experiments, a TCDD-dependent increase of *Hsp70.1* was observed, although this was not

marked. It is, therefore, likely that *Hsp70.1* induction by dioxins requires other, as yet, unknown factor(s).

Ciolino *et al*.[34] reported that the increase in CYP1A1 mRNA in MCF-7 cells produced by dimethylbenzanthracene (DMBA), a carcinogen and an AhR ligand, was partially antagonized by simultaneous treatment with curcumin. In addition, using an electrophoretic-mobility shift assay to measure the nuclear accumulation of activated AhR, they showed that curcumin could partially inhibit the activation of AhR by DMBA. However, curcumin has an ability to increase the accumulation of CYP1A1 mRNA as a ligand of AhR in MCF-7 cells. Thus, curcumin appears to act not only as an antagonist of AhR in the presence of other AhR ligands such as DMBA or TCDD, but also as a ligand of AhR. On the other hand, our study showed that curcumin has no effect on EROD activity in hepatic microsomes from TCDD-treated mice (Fig. 5). Although we were unable to explain this discrepancy, it may be attributable to the bioavailability and pharmacokinetics properties of curcumin. To date, several papers have described the absorption, tissue distribution and metabolism of [^{3}H]-curcumin after oral administration to rats. Measurements of plasma levels of radioactivity have shown that curcumin is moderately absorbed (approximately 60 to 66% of the given dose) from the gut.[35] A pharmacokinetic study using a dose of 80 mg showed that the total radioactivity in liver was less than 1% of the administered radioactivity over the period 0.5–24 hr following treatment.[36] Therefore, it should be noted that curcumin inhibited the induction of Cyp1a1 but did not accumulate in the liver in this study. However, even when we gave curcumin to mice in a higher dose (900 mg/kg/day × 5 days) than in the present study (100 mg/kg), we failed to detect any increase in hepatic EROD activity (data not shown). This observation suggests an alternative possibility, namely, that curcumin has no or only a minor ability to affect the activation of AhR-mediated gene regulation in mice *in vivo* in comparison with the MCF-7 cell system reported by other workers.[34]

In conclusion, we have shown that curcumin reduces adverse effects such as the reduction in body weight gain produced by TCDD. Although the mechanism is not yet fully understood, curcumin seems to exhibit the above effect without affecting AhR activation by TCDD. This study could not detect any target explaining the protective effect of curcumin, however, our results provide new insights into the development of therapeutic and preventive approaches involving dietary ingredients to combat dioxin toxicity.

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