Piperine, a Pepper Ingredient, Improves the Hepatic Increase in Free Fatty Acids Caused by 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin

Takumi Ishida, Mizuho Ishizaki, Siho Tsutsumi, Yuji Ishii, and Hideyuki Yamada*

Graduate School of Pharmaceutical Sciences, Kyushu University, 3–1–1 Maidashi, Higashi-ku, Fukuoka 812–8582, Japan

(Received June 5, 2008; Accepted July 26, 2008; Published online September 3, 2008)

Dioxins, such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), are ubiquitous environmental pollutants. The variety of adverse effects produced by dioxins are a serious problem because they may affect humans and wild animals through the food chain. In this study, we examined the possible protective effects of piperine, which is a major alkaloid in black pepper (*Piper nigrum* Linn.) and long pepper (*Piper longum* Linn.), on the toxic effects of TCDD in C57BL/6J mice. The repeated administration of high doses (30 and 45 mg/kg, 14 days, *p.o.*) of piperine alone produced a weak agonistic effect on the aryl hydrocarbon receptor, which was evaluated based on the increase in hepatic ethoxyresorufin *O*-deethylase (EROD) activity. No such effect was observed at the lowest dose (15 mg piperine/kg). However, while coadministration (20 mg/kg, 28 days, *p.o.*) of piperine with TCDD had no effect on TCDD-induced wasting syndrome, it improved the hepatic accumulation of free fatty acids produced by TCDD. In relation to this, the hepatic accumulation of triglycerides by TCDD also tended to be reduced by piperine. Despite the above effects, piperine failed to reduce the increase in hepatic EROD activity and lipid peroxidation produced by TCDD. These results suggest that piperine is a candidate to improve disorders of lipid metabolism produced by dioxins, although the mechanism remains to be clarified.

Key words —— piperine, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, steatosis, free fatty acid

INTRODUCTION

Dioxins are one of the most widespread, persistent, highly toxic environmental pollutants. It is well known that dioxins cause a variety of adverse effects in humans and wild animals including reproductive toxicity, teratogenicity, immune dysfunction, hepatotoxicity, and endocrine changes.[1–4] The activation of the aryl hydrocarbon receptor (AhR) by binding to dioxins is believed to be a key step in the production of these toxic effects.[5] It has also been suggested that the oxidative stress produced by dioxin plays a role in its toxic manifestations (see review by Ishida *et al.*[6]). In spite of this, the mechanism of dioxin toxicity remains largely unknown. In addition, some effects of dioxin which do not require AhR activation have been reported.[7] Such a complicated situation is one of the reasons why effective prophylaxis and treatment of the toxic effects of dioxin have not yet been developed.

The main route of dioxin ingestion by humans and wildlife is through food.[8] Several studies reported that absorption of dioxins *via* contaminated food accounts for more than 90% of the intake by adult humans.[9] Since environmental dioxins cannot easily be eliminated, the ingestion of those substances through the food chain is a continuous threat. Therefore it is important to develop a methodology that can combat the unavoidable ingestion of dioxins. One of the requirements for agents capable of combating dioxin toxicity is that they should be safe even if taken chronically. Some food ingredients, such as plant components, may have features that meet this requirement. Based on this concept, we have performed a series of studies that suggest that several polyphenols are promising candidates.[10–12] In the present study, we focused on piperine (1-piperoyl piperidine) (Fig. 1), which is a major alkaloid of black pepper (*Piper nigrum* Linn.) and long pepper (*Piper longum* Linn.). This compound has several pharmacologic activities such as antifungal,[13] anticancer,[14] hepatoprotective, and antioxidative effects.[14, 15] In addi-

*To whom correspondence should be addressed: Graduate School of Pharmaceutical Sciences, Kyushu University, 3–1–1 Maidashi, Higashi-ku, Fukuoka 812–8582, Japan. Tel.: +81-92-642-6585; Fax: +81-92-642-6588; E-mail: yamada@xenoba.phar.kyushu-u.ac.jp

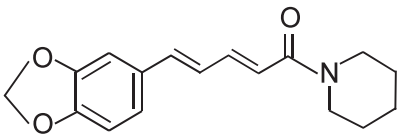

Fig. 1. Chemical Structure of Piperine

tion, Amakura *et al.*[16] have reported an antagonistic effect of piperine on 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD)-induced AhR activation *in vitro*. Due to these characteristics, piperine is also expected to antagonize the toxic effects of dioxin *in vivo*. Thus we have examined the effects of piperine on the acute toxicity of TCDD in mice.

MATERIALS AND METHODS

Reagents —— TCDD (purity > 99%) was obtained from AccuStandard, Inc. (New Heaven, CT, U.S.A.). A stock solution was prepared by dissolving TCDD in acetone (40 μg/ml) and this was stored at −20°C until use. Nicotinamide adenine dinucleotide phosphate (NADPH), 7-ethoxyresorufin, and thiobarbituric acid were purchased from Sigma (St. Louis, MO, U.S.A.). Piperine (97% purity) and resorufin sodium salt were obtained from Aldrich Chemical Co., Inc. (Milwaukee, WI, U.S.A.). 1,1,3,3-Tetraethoxypropane was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). All other reagents were of analytical grade and commercially available.

Animals and Treatments —— All experiments were approved by the Institutional Animal Care and Experiment Committee of Kyushu University. TCDD stock solution was diluted with corn oil to give a concentration of 20 μg/ml, and the acetone was evaporated under nitrogen. Piperine was suspended in 0.5% methylcellulose to give a concentration of 4 mg/ml, and both solutions were kept in the dark until use and were then vortex-mixed before administration. Male C57BL/6J mice (5 weeks old, CLEA Japan, Tokyo, Japan) were randomly divided into groups of 5 or 6 mice and given oral piperine (20 mg/kg body weight/5 ml) or vehicle. The dose of piperine was decided on the basis of our preliminary experiments (see Results section). Mice were then given TCDD orally (100 μg/kg body weight/5 ml) or vehicle 90 min after piperine administration. After the treatment described above on day 0, piperine was administered once a day at the same dose for the next 28 days. During the study, mice were allowed access to food and water *ad libitum* and were weighted before administration. Twenty-four hours after the last treatment, organs from all the mice were removed and weighed. Hepatic homogenate (10%) was prepared with 1.15% KCl, and an aliquot was centrifuged at 9000 × *g* for 20 min to prepare a supernatant as a source of cytochrome P450 (Cyp). All prepared samples were stored at −80°C until use.

Measurement of Hepatic Triglyceride and Free Fatty Acid (FFA) Concentrations —— The hepatic concentrations of triglycerides and FFAs were measured based on a color reaction using acyl-CoA oxidase[17] and glycerol-3-phosphate oxidase,[18] respectively. Triglycerides and FFAs were extracted from 75 μl of hepatic homogenate with 1 ml of isopropyl alcohol. The extraction was carried out by vortex-mixing for 10 min, and then centrifuging at room temperature and 800 × *g* for 5 min. After centrifugation, 300 μl samples of the supernatant were transferred to two test tubes, followed by determination of FFAs and triglycerides. The measurements were performed according to manufacturer's protocol supplied with the commercial kits, NEFA C-Test WAKO and Triglyceride E-Test WAKO (Wako Pure Chemical Industries, Ltd.).

Other Methods —— The activity of hepatic ethoxyresorufin *O*-deethylase (EROD) and the concentration of thiobarbituric acid-reactive substances (TBARS) were measured using the methods reported previously.[10] Statistical significance was calculated using Fischer's protected least significant difference test.

RESULTS

Toxic Effects of Piperine and Its Effects on AhR Signaling

To examine the toxicity of piperine itself, it was given orally to male C57BL/6J mice at doses of 15, 30, and 45 mg/kg. Following the initial administration on day 0, mice were treated once a day with the same dose for 14 days, and their organs were removed 24 hr after the last administration. The effects of piperine on body weight gain are shown in Fig. 2. During the experiment, the body weights of all the treated groups increased daily. However, a significant reduction compared with the control was observed in the 45 mg/kg group from day 9. The highest dose group also showed a significant reduction in spleen and thymus weights, whereas the liver

weight remained unaffected (Table 1). When we focused on EROD activity, a marker of AhR activation, it was increased by about 2.5-fold compared with the control group following the administration of 30 and 45 mg/kg of piperine (Fig. 3A). In contrast, piperine treatment did not have any significant effect on the hepatic TBARS concentration, although the value tended to be reduced in the groups given 15 and 30 mg/kg (Fig. 3B). Thus, since piperine seems to have a weak agonistic effect on AhR signaling, the coadministration experiments with TCDD shown below were conducted using piperine 20 mg/kg, which was assumed to be close to the maximal dose to avoid toxicity and its effects on the AhR.

Effects of Piperine on TCDD-Induced Wasting Syndrome

The effects of piperine on the changes in body weight induced by TCDD are shown in Fig. 4. A reduction in body weight gain was observed in the TCDD-treated group compared with control from day 1. While the same sign was observed in the piperine + TCDD-treated group, this group tended to recover from day 18. Piperine coadministration had little effect on liver hypertrophy and thymic and splenic atrophy produced by TCDD (Table 2).

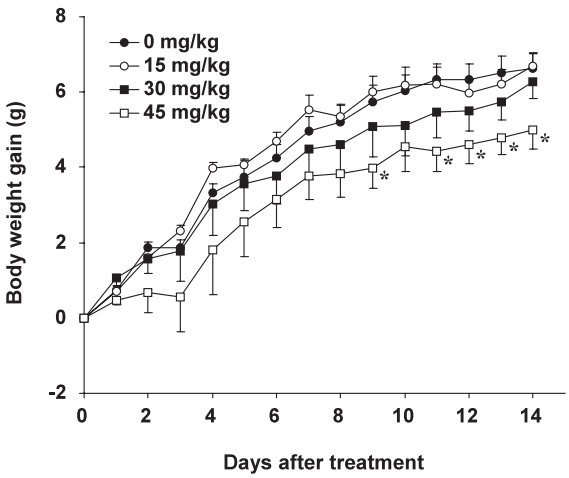

Fig. 2. Changes in the Body Weight of C57BL/6J Mice Following Exposure to Piperine

The plots represent the mean ± S.E. The initial body weights (mean ± S.E.) of mice were 14.7 ± 1.2 (control), 15.1 ± 0.8 (piperine 15 mg/kg), 14.6 ± 0.4 (30 mg/kg), and 15.1 ± 0.3 g (45 mg/kg), respectively. *Significantly different from control ($p < 0.05$).

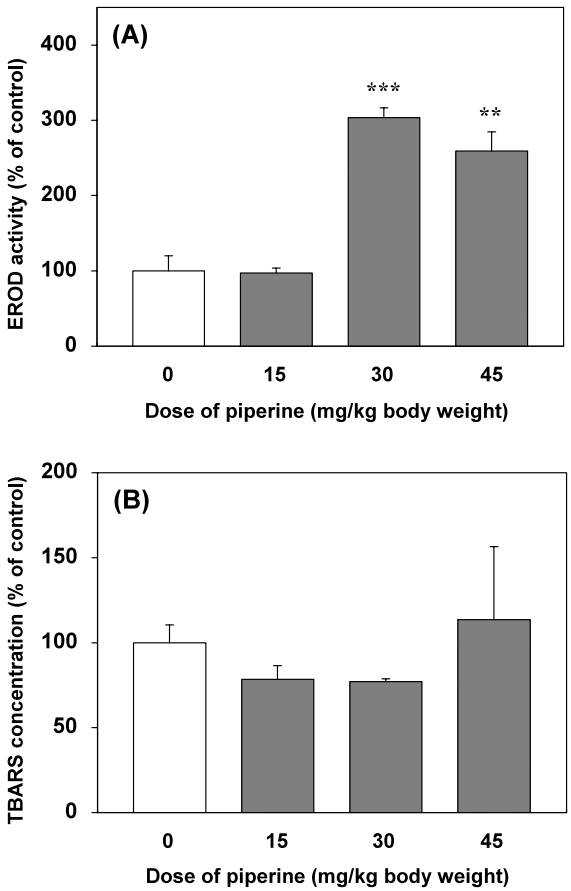

Fig. 3. Effects of Piperine on Hepatic EROD Activity (A) and TBARS Concentration (B) in C57BL/6J Mice

The values represent the mean ± S.E. The EROD activity and TBARS concentration in the control group were 1.19 ± 0.23 pmol/min/mg protein and 222 ± 23 nmol/g liver, respectively. Significantly different from control: **$p < 0.01$, ***$p < 0.001$.

Table 1. Effects of Piperine on the Change in Organ Weights of C57BL/6J Mice

Dose	Liver	Spleen	Thymus
	(% of body weight)		
Control	5.71 ± 0.34	0.31 ± 0.01	0.30 ± 0.02
15 mg/kg	5.45 ± 0.32	0.31 ± 0.02	0.25 ± 0.01*
30 mg/kg	5.72 ± 0.07	0.29 ± 0.03	0.25 ± 0.02
45 mg/kg	5.81 ± 0.10	0.23 ± 0.01***	0.19 ± 0.01***

The values represent the mean ± S.E. of 5 mice. Significantly different from control: *$p < 0.05$, ***$p < 0.001$.

Table 2. Effects of Piperine on the Change in Organ Weights of C57BL/6J Mice after Exposure to TCDD

Treatment	Liver	Spleen	Thymus
	(% of body weight)		
Control (5)	5.17 ± 0.11	0.25 ± 0.01	0.17 ± 0.02
Piperine (5)	5.08 ± 0.10	0.25 ± 0.01	0.13 ± 0.01*
TCDD (6)	7.90 ± 0.36**	0.21 ± 0.01**	0.03 ± 0.01***
Piperine+TCDD (5)	8.20 ± 0.24***	0.23 ± 0.02	0.04 ± 0.01***

The values represent the mean ± S.E. of 5 or 6 mice. The number of samples is shown in parentheses. Significantly different from control: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

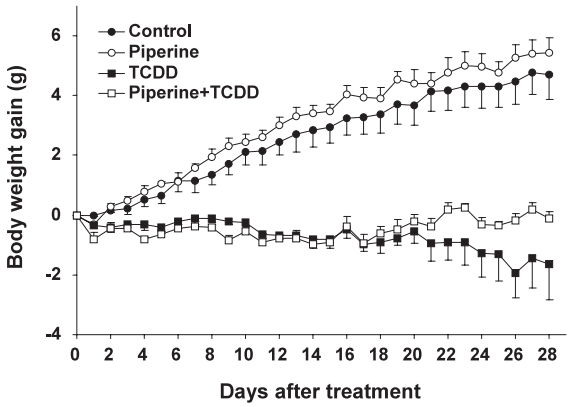

Fig. 4. Changes in the Body Weight of C57BL/6J Mice Following Exposure to Piperine and TCDD

Mice pretreated with piperine 20 mg/kg were treated once with TCDD on day 0. The mice were then given the same dose of piperine daily for the next 28 days. The plots represent the mean ± S.E. of 5 or 6 mice. The initial body weights (mean ± S.E.) of mice in the control, and piperine-, TCDD- and piperine + TCDD-treated groups were 19.9 ± 0.3, 19.9 ± 0.2, 19.7 ± 0.2 and 19.7 ± 0.4 g, respectively.

Effects of Piperine on TCDD-Induced Lipid Accumulation in the Liver

We then examined the effects of piperine on the TCDD-induced accumulation of hepatic FFAs and triglycerides. As reported,[19,20] the concentrations of hepatic FFAs and triglycerides in the TCDD group were significantly increased (Fig. 5). However, coadministration with piperine markedly improved the increase in FFAs induced by TCDD (Fig. 5A). In connection with this, piperine coadministration also showed a tendency to reduce hepatic triglycerides, although this was not significant (Fig. 5B).

Effects of Piperine on AhR Activation and Oxidative Stress Produced by TCDD

To investigate whether piperine modifies either AhR activation or lipid peroxidation by TCDD to improve FFA accumulation, the effects of piperine on the TCDD-induced increase in hepatic EROD activity and TBARS concentration were determined.

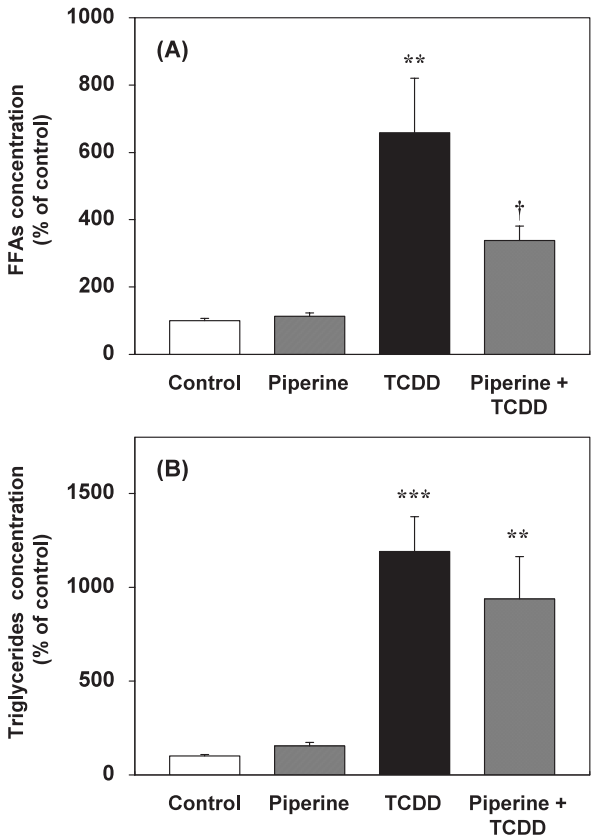

Fig. 5. Effects of Piperine on the Hepatic Concentrations of FFAs (A) and Triglycerides (B) in C57BL/6J Mice Following Exposure to TCDD

Bars represent the mean ± S.E. of 3 mice. The concentrations of FFAs and triglycerides in the control group were 0.90 ± 0.06 μEq/g liver and 4.24 ± 0.38 mg/g liver, respectively. Significantly different from control: $^{**}p < 0.01$, $^{***}p < 0.001$; from TCDD-treated group: $^{\dagger}p < 0.05$.

As can be seen in Fig. 6, TCDD significantly enhanced both EROD activity and TBARS concentration. In contrast, coadministration of piperine did not have any marked effect on these changes.

DISCUSSION

In the present study, we investigated the ef-

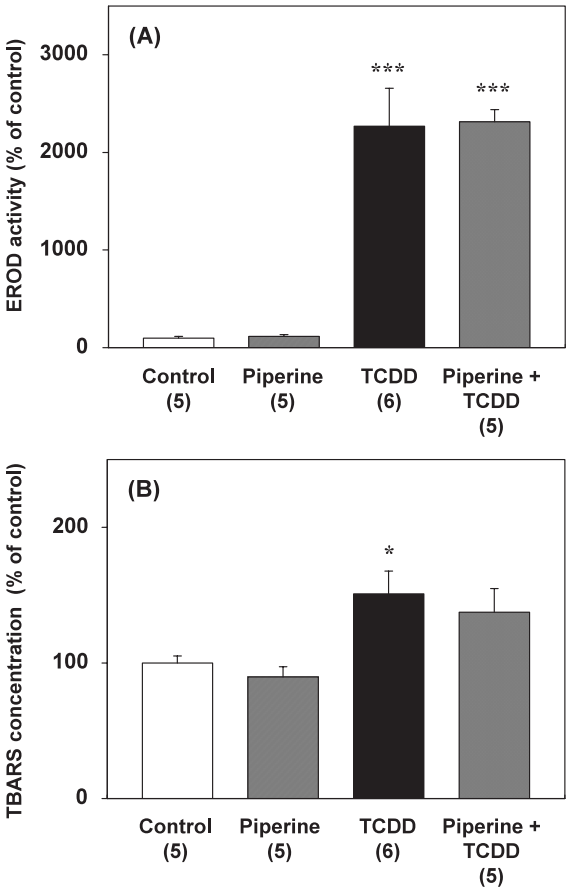

Fig. 6. Effects of Piperine on Hepatic EROD Activity (A) and TBARS Concentration (B) of C57BL/6J Mice Following Exposure to TCDD

Bars represent the mean ± S.E. of 5 or 6 mice. The number of samples is shown in parentheses. The EROD activity and TBARS concentration in the control group were 4.02 ± 0.61 pmol/min/mg protein and 239 ± 12 nmol/g liver, respectively. Significantly different from control group: $^{*}p < 0.05$, $^{***}p < 0.001$.

fects of piperine on the acute toxicity of TCDD. Our results showed that piperine had no effect on wasting syndrome including the changes in organ weight produced by TCDD. In addition, EROD activity increase induced by TCDD was also unaffected following coadministration of piperine. It has been established that hepatomegaly, thymic atrophy, and wasting syndrome including the loss of body weight occur through an AhR-dependent mechanism.[5, 21, 22] The same toxic signs were also reproduced in this study. While piperine has been reported to have only an antagonistic effect against the TCDD-induced activation of AhR *in vitro*,[16] the same was not true in our *in vivo* study. The reason for this inconsistency remains unknown. However, it is widely accepted that a number of plant ingredients in food have poor bioavailability due to their rapid metabolism. For example, resveratrol and its metabolites disappear from serum within 2 hr following the oral administration of this polyphenol to mice.[23] Although the pharmacokinetics of piperine is still unclear, the enzyme sources for the assay of EROD activity were prepared 24 hr after the last administration of piperine in this study. Therefore, one possibility is that the effects of piperine on AhR activation disappeared following its metabolism. Another possibility is that the toxic and biological effects produced by TCDD overcame the protective effects of piperine under our experimental conditions. The dose of TCDD (100 μg/kg) used was almost the half the LD_{50} for C57BL mice.[21] Therefore, the mice might have had extensive damage due to the high dose of TCDD, in agreement with the above possibility. Increasing the piperine dose may solve this problem. However, our results also suggest that piperine itself is a potent agonist of the AhR at doses greater than 30 mg/kg. A loss of body weight gain, atrophy of the spleen and thymus, and an increase in hepatic EROD activity were observed in the piperine 45 mg/kg group (Figs. 2 and 3A, Table 1). To the best of our knowledge, no study has reported an agonistic action of piperine on the AhR. Therefore, even if piperine is really an AhR agonist, it should be clarified whether this alkaloid directly binds to the AhR. Based on the data obtained here, it should be noted that increasing the piperine dose may be risky due to the enhancement of dioxin toxicity produced by the additive activation of the AhR.

The accumulation of triglycerides and/or cholesterol in hepatocytes, which is considered a manifestation of steatosis, is one of the typical biological responses seen after exposure to dioxins.[19, 20] Although the mechanism of dioxin-induced steatosis is still unclear, it is suggested that it is caused by mechanisms including an increase in lipid uptake by hepatocytes and/or dysfunction of lipid metabolism.[19, 24–26] It is also assumed that the oxidative stress produced by TCDD is involved in the production and progression of steatosis, because a positive link between oxidative stress and steatosis has been demonstrated (see the review by Mantena *et al.*[27]). The present study provides the first evidence that piperine has a beneficial effect on the increase in the concentrations of hepatic FFAs and triglycerides produced by TCDD. However, it remains unclear whether piperine is able to modulate the expression and/or function of proteins contributing to lipid metabolism and lipid intake. Keeping this in mind, the antioxidative effect of piperine[14, 15] may provide a clue to understanding

the mechanism whereby this substance improves lipid status. As mentioned above, oxidative stress is assumed to be one of the major factors capable of promoting steatosis. This would support the above view. However, our results show that the coadministration of piperine fails to improve the hepatic TBARS concentration increased by TCDD. Although we were unable to clarify this discrepancy, the effect of piperine may have disappeared due to metabolism, because the source of TBARS was prepared 24 hr after the last administration of piperine. Thus it cannot be excluded that the repeated administration of piperine antagonizes the oxidative stress induced by TCDD, although the effect is not marked.

It is well established that FFAs transported into the liver are metabolized by mitochondrial and peroxisomal β-oxidation and Cyp4a-catalyzed ω-oxidation.[28,29] Some critical enzymes involved in these reactions are believed to be transcriptionally controlled by peroxisome proliferator-activated receptor α (PPARα).[30] This is supported by the observation that the constitutive expression of enzymes involved in mitochondrial and peroxisomal fatty acid β-oxidation, such as long-chain acyl-CoA dehydrogenase, are significantly lower in PPARα-knockout mice than in wild-type mice.[31] This suggests that the disruption of PPARα signaling is one of the driving forces for the onset of steatosis. If this is true, it would be reasonable to assume that the activation of PPARα signaling leads to the attenuation of this steatosis. It has been reported that the coadministration of di(2-ethylhexyl)phthalate, the metabolites of which are thought to be the PPARα ligands, reduces the hepatic accumulation of lipids induced by TCDD.[32] Thus another possibility is that piperine or its metabolites attenuates the accumulation of hepatic FFAs induced by TCDD by activating PPARα signaling. It is unclear whether piperine or its metabolites is able to bind to PPARα as a ligand. However, the PPARα ligand has been reported to downregulate the expression of the UDP-glucose dehydrogenase (UGDH) gene.[33] Since piperine has also been reported to reduce UGDH mRNA and its activity *in vivo* and *in vitro*,[33,34] these observations would support the above hypothesis.

Our present study suggests that piperine has a beneficial effect on dioxin-induced steatosis. However, as mentioned previously, it should be kept in mind that excess piperine increases the toxic effects of dioxin because of its own weak agonistic action. Further studies, including clarification of the mechanisms involved in the biological action of piperine, are needed to address this issue.

Acknowledgement This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Health, Labour, and Welfare, Japan.

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