Enhancement of Dioxin Toxicity with an Anti-Stress Drug, Carbenoxolone, in Mice

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The effect of the carbenoxolone (CBX) on the subacute toxicity of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) was studied in C57BL/6J mice. The loss of body weight due to TCDD was enhanced by simultaneous treatment with CBX. In agreement with the change in body weight, CBX failed to improve TCDD-induced hepatic hypertrophy and thymic atrophy. Co-treatment of CBX with TCDD tended to cause hepatic hypertrophy more extensively than did TCDD alone, and combined treatment induced significant renal hypertrophy and splenic atrophy which were not seen in mice treated with TCDD or CBX alone. CBX had no effect on the TCDD-mediated induction of hepatic ethoxyresorufin *O*-deethylase activity, a marker for aryl hydrocarbon receptor (AhR)-linked gene expression. These results suggest that CBX enhances TCDD toxicity by a mechanism(s) distinct from activation of the AhR.

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

INTRODUCTION

Dioxins, including 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and related compounds, are one of the most widely distributed environmental pollutants. Because of their lipophilicity, stability and resistance to biodegradation, dioxins accumulate in the food chain and are retained in human adipose tissue. TCDD is known to be most toxic of all dioxins and produces a wide spectrum of effects including death, wasting syndrome, atrophy of the thymus and spleen, tumor promotion, immunosuppression, teratogenicity, and endocrine changes.[1–4] Although dioxins are known to exert their toxic effect through interaction with the aryl hydrocarbon receptor (AhR),[5–7] the mechanism of dioxin toxicity following AhR activation has not been elucidated.

In a series of our studies to clarify the toxic mechanism of dioxins, we found that heat shock protein 70 (HSP70) is induced in the hepatic cytosol of rats treated with a dioxin-like compound, 3,3′,4,4′,5-pentachlorobiphenyl (PCB, IUPAC No. PCB126).[8] HSP70 is one of the intracellular chaperons which play an important role in the main-

tenance of functional proteins.[9] The expression of HSPs is known to be increased in response to a variety of stresses such as heat shock, hypoxia, hydrogen peroxide, inflammation, and ischemia.[10] Among the various HSP isoforms, HSP70 is the predominant one that is induced by a variety of stresses. Therefore, it would be resonable to hypothesize that the living body induces HSP70 in response to PCB126 stress in order to avoid lethal damage by the dioxin.

Carbenoxolone (CBX, Fig. 1), a glycyrrhizic acid derivative, protects the gastric mucosa against a variety of noxious agents in experimental animals and accelerates the healing of peptic ulcer in man without inhibiting acid secretion.[11–13] Although increased glycoprotein synthesis,[14] inhibition of enzymes that inactivate prostaglandins,[15] and suppression of the activation of pepsinogen[16] have been suggested as mechanisms of CBX action, a recent study has demonstrated that this drug specifically induces HSP70 expression, by a mechanism involving heat shock factor 1 activation.[17] Since the induction of HSP70 by several drugs and mild heat shock protects gastric mucosal cells,[18–21] it is likely that the induction of HSP70 by CBX contributes to this therapeutic effect.

In the light of the findings mentioned above, we have carried out a study to see whether CBX, an HSP70 inducer, reduces TCDD toxicity in mice. However, the results did not support the antagonis-

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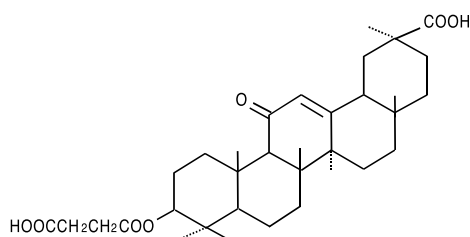
Fig. 1. Structure of CBX

Fig. 2. Change in the Body Weight of C57BL/6J Mice after Exposure to a Single Dose of TCDD and/or Admistration of CBX

The body weight of mice at the start of the experiment was 20 ± 0.78 g. Each plot represents the mean $\pm$ S.E. of surviving mice ($n = 5$–8). Statistical significance was calculated by Scheffe's test in CBX + TCDD treatment group. Significantly different from the control: *$p < 0.05$. Significantly different from TCDD treatment group: †$p < 0.05$. The open and closed arrow heads show the day when mice died in the TCDD treatment group and CBX + TCDD treatment group, respectively.

tic effect of CBX on TCDD toxicity. This paper describes the finding that the simultaneous treatment of C57BL/6J mice with CBX and TCDD enhanced the effect on the loss of body weight by TCDD, and this effect of CBX is suggested not to be due to the effect on AhR signaling.

MATERIALS AND METHODS

Reagents —— TCDD (purity > 99% as determined by gas chromatography-mass spectrometry) was obtained from AccuStandard, Inc. (New Haven, CT, U.S.A.). TCDD was dissolved in acetone at a concentration of 40 μg/ml, and stored at –20°C. When needed, the above solution was diluted 2.4 times with corn oil, and the acetone was evaporated under nitrogen gas. CBX was donated by Tokiwa Phytochemical Co. Ltd. (Chiba, Japan). The drug was stored at 4°C in the dark and dissolved in water before use. All other chemicals were of analytical grade commercially available.

Animals and Treatments —— Male C57BL/6J mice (4 weeks old) purchased from CLEA Japan Inc. (Tokyo, Japan) were acclimatized for one week prior to treatment. Mice were randomly assigned to treatment groups by body weight. Eight or five mice per group were housed in cages (5 mice in the control and CBX treatment groups and 8 mice in the TCDD and CBX + TCDD treatment group). On day 0, mice were given CBX or the same volume of the vehicle with oral administration at a dose of 100 mg/kg body weight/4 ml. Then, 30 min after CBX treatment, TCDD or the same volume of the vehicle was given once at a dose of 100 μg/kg/6 ml corn oil. CBX or the same volume of the vehicle was continuously administered at the same dose once a day. One day after the last administration, the organ weights of all treated mice were measured, and hepatic microsomes were prepared by successive centrifugation of liver homogenate.
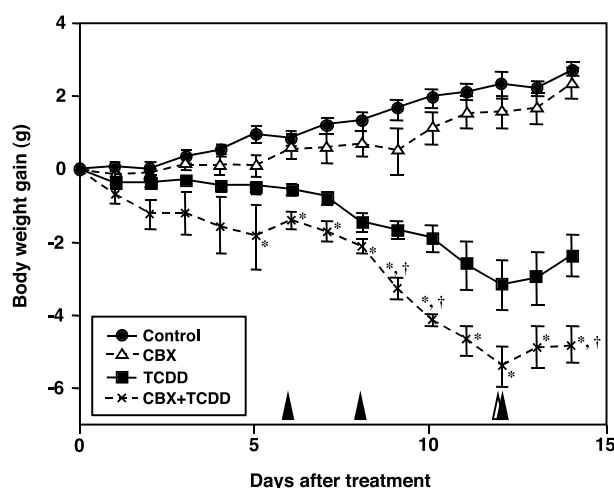
Other Methods —— The protein content of microsomes was determined according to the method of Lowry *et al.*[22] with bovine serum albumin as a standard. The ethoxyresorufin O-deethylase (EROD) activity in hepatic microsomes was determined by the method of Burke and Mayer.[23] Briefly, 20 nM ethoxyresorufin was incubated for 3 min in an incubation solution (1.5 ml) containing hepatic microsomes (0.1 mg protein), 2 mM NADPH and 0.1 M Tris–HCl (pH 7.8). The fluorescence of resorufin, a deethylated metabolite, was monitored at 585 nm (exitation, 530 nm).

RESULTS

Effect of CBX on TCDD-Induced Toxicity

The effect of CBX on the changes in body weight gain by TCDD is shown in Fig. 2. In mice treated with vehicle or CBX alone, the body weight increased day by day. On day 14, the means of the percentage body weight gain in the control and CBX treatment groups were approximately 14 and 12%, respectively. In contrast, the body weight decreased in mice treated with TCDD. The mean of the percentage decrease at day 14 was approximately 12%. Interestingly, a severe decrease in body weight was

Table 1. Change in Organ Weights of C57BL/6J Mice Following Treatment with TCDD and CBX

organ	Liver	Thymus	Spleen	Kidney
	(% of body weight)			
control (5)	5.54 ± 0.05	0.22 ± 0.01	0.31 ± 0.04	1.35 ± 0.02
CBX (5)	$6.72 \pm 0.08^*$	0.22 ± 0.01	0.29 ± 0.01	1.28 ± 0.02
TCDD (7)	$7.07 \pm 0.19^*$	$0.02 \pm 0.00^{**}$	0.24 ± 0.01	1.43 ± 0.04
CBX + TCDD (5)	$7.72 \pm 1.28^{**}$	$0.02 \pm 0.01^{**}$	$0.17 \pm 0.10^*$	$1.94 \pm 0.45^{**}$

The values represent the mean $\pm$ S.E. of surviving mice. The number of the sample is shown in parenthesis. Significantly different from control: $^*p < 0.01$; $^{**}p < 0.001$.

observed in mice co-treated with CBX and TCDD. Significant differences in body weight between two groups (TCDD vs. CBX + TCDD) were detected at days 9, 10 and 14. On day 14, the body weight in the CBX + TCDD treatment group was decreased by approximately 24% in comparison with the initial weight. Table 1 shows the effects of TCDD and/or CBX on organ weights. As expected, a significant increase in liver weight and atrophy of the thymus were observed following TCDD treatment, although CBX also increased liver weight. In agreement with the change in body weight, simultaneous treatment of CBX and TCDD tended to increase hepatic hypertrophy more markedly than TCDD alone, though it was not significant. Significant symptoms of renal hypertrophy and splenic atrophy were seen only in the simultaneous treatment group.

Effect of CBX on TCDD-Mediated Gene Expression

Dioxins are known to exert their toxicity by an alteration of gene expression through AhR activation.[7,24,25] Therefore, synergism by CBX in TCDD toxicity may be due to enhancement of the TCDD-mediated activation of AhR. To address this issue, we next examined the effect of CBX on hepatic EROD activity which is catalyzed by CYP1A1, a well-known enzyme inducible by TCDD-mediated activation of AhR. The results (Fig. 3) showed that the degree of induction of hepatic EROD activity was comparable in mice treated with TCDD alone and CBX + TCDD, and no significant difference was seen between the two groups. Although the effect of CBX on early phases of EROD induction by TCDD (endpoint of above data shown was day 14 after continuous CBX exposure) is unknown, the data shown in Fig. 3 suggested that CBX enhances TCDD toxicity by a mechanism(s) not involving AhR activation.
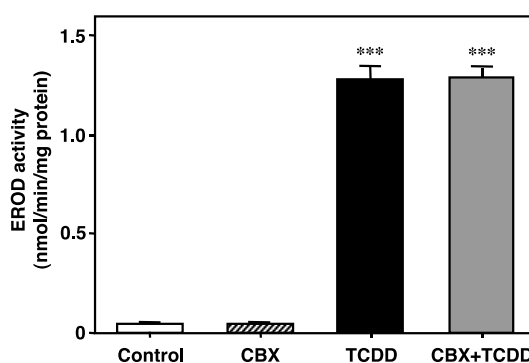

Fig. 3. Effect of Carbenoxolone on Hepatic EROD Activity of C57BL/6J Mice Treated with TCDD

The EROD activity of the control was 0.034 ± 0.005 nmol resorufin formed/min/mg protein. Statistical significance was calculated by Scheffe's test. Significantly different from control: $^{***}p < 0.001$. The values represent the mean $\pm$ S.E. ($n = 5$).

DISCUSSION

In the present study, we examined the effect of CBX used to treat peptic ulcer on the toxicity of TCDD. We wished to know whether enhancement of the anti-stress system results in a reduction in TCDD toxicity. However, CBX enhanced wasting syndrome by TCDD (Fig. 2). Although the mechanism underlying the stimulation of dioxin toxicity by CBX remains to be clarified, it is suggested that the synergism results from the specific effects of CBX which differ from those of TCDD. Because CBX itself causes hepatomegaly (Table 1), it may be conceivable that the increase of wasting syndrome by co-treatment with TCDD and CBX is due to the additive effects of these compounds. However, the observation that CBX showed no significant difference on body weight supports at least partially the synergistic effect of this compound on TCDD-mediated wasting syndrome.

CBX is known to be a potent inhibitor of 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2). This enzyme is abundantly expressed in the placenta

and fetus,[26] and plays a role in converting corticosterone to inert 11-dehydrocorticosterone.[27] In accordance with this, CBX reduces placental 11β-HSD2 activity and increases corticosterone levels in fetal tissues.[28] Therefore, it would be reasonable to expect that the CBX-caused increase in active corticosterone has an additional effect to the toxicity of TCDD. Although a synergistic interaction between TCDD and glucocorticoid resulting in teratogenesis has been reported to be caused by low doses,[29] Taylor et al.[30] provided evidence that dexamethasone, a synthetic glucocorticoid, reversed the acute toxicity of TCDD in mice. They also showed that the decrease in body weight 11 days after TCDD + dexamethasone treatment was approximately 3%, whereas that after treatment with TCDD alone was approximately 15%. In addition, dexamethasone treatment reduced the mortality of TCDD by 92%. If an increased level of glucocorticoid indeed rescues mice from TCDD toxicity, the CBX-induced synergism in TCDD toxicity is likely to be due to a reason other than 11β-HSD2 inhibition. This is because inhibition of this enzyme would elevate the level of active glucocorticoid.

Damage to gap junction intercellular communications (GJIC) by CBX and TCDD is also proposed to explain the CBX-TCDD synergism observed in this study. Cellular homeostasis is regulated by the passage of low-molecular-weight molecules, such as neurotransmitters, ATP, Ca^{2+} and hormones, through transmembrane channels in a process called GJIC.[31,32] Gap junctional proteins, connexins, allow the intercellular passage of both negative and positive homeostatic elements, suggesting a prominent role for GJIC in cell growth, differentiation, embryogenesis, and neoplastic development.[33] TCDD down-regulates GJIC in rat hepatocytes in a concentration- and duration-dependent manner at noncytolethal concentration ranges.[34] The above study showed that treatment of rat hepatocytes with TCDD resulted in a decrease in connexin32 mRNA without any apparent effect on connexin26 mRNA.[35] The effect of TCDD on connexin32 seems to require AhR activation. Furthermore, TCDD failed to inhibit GJIC function in the rat liver-derived cell line WB F344, which primarily expresses connexin43 along with a lesser amount of connexin26 and does not express connexin32 at all.[35] From these results, it appears that connexin32 is one of selective factors in the effects of TCDD. On the other hand, CBX is reported to block GJIC by inhibition of connexin43 without decreasing the expression of gap junction.[36]

This report also described that glycyrrhetinic acid derivatives can completely and reversibly inhibit transfer of calcein and carboxyfluorescein between connexin43-transfected C6 glioma cells in a noncytotoxic manner without significantly affecting their growth rates. The mechanism of this inhibition was proposed as follows: glycyrrhetinic acid derivatives bind directly to the gap junction, inducing a conformational change and subsequent channel closure.[37] Taking the above information into consideration, TCDD and CBX seem to reduce the GJIC function by impairing connexin32 and 43, respectively. Isenberg et al.[38] have suggested that the inhibition of GJIC function contributes to the increase in the liver weight of B6C3F1 mice by di-2-ethylhexylphthalate. The reverse correlation between the GJIC function and liver weight has also been observed in rats, while hamsters do not exhibit any such correlation.[38] Thus, the dysfunction of GJIC is suggested to be one of the mechanisms in drug-induced liver hypertrophy, although the significance of this mechanism differs from species to species. The relationship between enhanced toxicities with co-treatment of CBX with TCDD and the impairment of GJIC function is not clear. It is, however, possible that the synergism in TCDD/CBX-induced toxicity is due to the summation of the different effects of both compounds described above.

In summary, treatment with CBX enhanced the toxicity of TCDD in C57BL/6J mice. This effect was considered to be due to a mechanism distinct from the AhR-linked alteration in induction of EROD activity. Possible involvement of the CBX effects on GJIC in the enhancement of TCDD toxicity was discussed. Further studies are needed to clarify the mechanism(s) governing the synergistic effect of CBX.

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