Biochemical Pharmacology, Vol. 53, pp. 1397–1404, 1997.

ISSN 0006-2952/97/$17.00 + 0.00
PII S0006-2952(97)00036-1

RAPID COMMUNICATION

ALTERED *IN VIVO* TOXICITY OF 2,3,7,8-TETRACHLORODIBENZO-*p*-DIOXIN (TCDD) IN C-SRC DEFICIENT MICE

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Abstract. Administration of a single i.p. dose of 115 μg/kg of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) to homozygous and heterozygous c-src deficient mice (i.e. c-src -/- and -/+ mice) and their wild-type littermates (c-src +/+ mice) induced differential toxic responses. In c-src +/+ mice, there were clear-cut signs of the toxicity of TCDD, such as the loss of weight in the body, thymus and adipose tissue, whereas in c-src -/+ mice these effects were modest and were not statistically significant. Yet, hepatomegaly, a characteristic effect of TCDD, took place in all three strains of mice. Histological examination of liver samples from control mice and from mice treated with TCDD for 10 days showed that there are qualitative differences in the expression of the effects of TCDD between control and treated mice as well as between c-src -/+ and +/+ mice. In the case of c-src +/+ mice, the predominant lesions were lipid accumulation, glycogen depletion, edema formation and necrosis, as shown by the presence of large areas of ballooning degeneration, and cellular influx of fluid. These changes were demonstrated only marginally in c-src -/+ mice. The predominant effect in -/+ mice was edema formation. At a high dose of TCDD (345 μg/kg), all of the +/+ mice died within 34 days, whereas none of the c-src -/+ mice died. Together these results clearly indicate that some of the toxic effects of TCDD are not fully expressed in c-src deficient mice. BIOCHEM PHARMACOL **53**;10: 1397–1404, 1997. ©1997 Elsevier Science Inc.

Key words. TCDD; c-src deficient mice; reduction of toxicity; qualitative difference in hepatotoxicity

INTRODUCTION

TCDD is said to be the most toxic synthetic chemical known to man. It is also the most potent member of a broad class of chemicals called "dioxin-type chemicals" [1-3]. The well-known aspect of the action pathway of TCDD is that it initially binds with its high affinity receptor called the Ah

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Abbreviations: TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; and PAS, Periodic Acid Schiff.

Accepted 27 February 1997.

receptor, which translocates into the nucleus where it is processed through dimerization with ARNT and transactivates specific genes such as *CYP1A1* [4-6]. Largely unexplained, however, are the mechanisms by which TCDD actually causes a variety of *in vivo* toxic symptoms. Among various hypotheses, the best studied is the direct gene activation theory [see, for example, Refs. 7 and 8]. It is certainly likely that critical genes are directly activated in this manner. A hypothesis that we have proposed and are in the process of testing is a "phosphorylation pathway" theory [9, 10] in which the action of TCDD to activate protein tyrosine kinases, particularly c-Src kinase [11], is suggested to be responsible for some of the expression of the toxic endpoints of TCDD. To obtain further supporting evidence for the latter theory, we have decided to study the effect of TCDD in c-src -/- and -/+ mice, the product of c-src knockout treatment [12]. We found that in such mice certain expressions of the toxicity of TCDD were either reduced or eliminated as compared to those observed in wild-type mice, c-src +/+ littermates.

MATERIALS AND METHODS

Materials. TCDD was obtained originally from the Dow Chemical Co. (Midland, MI) as described previously [13]. All mouse strains were obtained from Jackson Laboratory (Bar Harbor, ME). They were B6, 129-Src^{tm1sor} (stock no. J2381) mice, consisting of homozygous c-src -/-, heterozygous -/+, and wild-type littermate controls, c-src +/+ mice. A preliminary study established that c-src -/- and -/+ mice express approximately 0 and 14.5% of c-Src kinase activity associated with the Ah receptor in hepatic cytosol homogenate as compared with that (= 100%) of the c-src +/+ wild strain. Only young males were studied. Their body weight ranges at the time of testing are shown in Table 1. Five mice were used for each batch of controls and treatments.

Treatments. Mice were intraperitoneally injected (i.p.) with 115 or 345 μg/kg of TCDD dissolved in a mixture of corn oil and acetone (9:1). Food and water were given *ad lib.*, and the body weight and the amount of food consumed were measured daily. At the end of a 10-day observation period, the 115 μg/kg TCDD-treated and the control mice were killed, and various organs were isolated and weighed. The high dose (345 μg/kg) treatment groups were kept for observation. The body weight and the mortality were recorded. The surviving individuals are still kept.

Histological Methods. Mouse liver tissue slices (1-2 mm thick) were immersion-fixed with 2% glutaraldehyde, in 0.085 M sodium cacodylate with 0.05% calcium chloride. Tissues were dehydrated through a series of graded ethanols and embedded in Immunobed resin (Polysciences Inc., Warrington, PA). Sections (1.5 μm thick) were cut and stained with methylene blue-basic fuchsin for routine examination, or with PAS stain for identification of glycogen deposits. Liver tissues examined for lipid deposits were embedded directly in plastic resin, and sections were stained with Nile Red, a lipid-specific stain (Sigma Chemical Co., St. Louis, MO) [14,15]. All sections were examined on a Zeiss Axioskop in bright field or epifluorescence at an excitation wavelength of 450-490 nm and an emission wavelength of 520 nm.

RESULTS

The effects of TCDD administered *in vivo* (115 μg/kg, single i.p. injection) on the weights of selected organs and the body were assessed at day 10 of post-treatment in homozygous c-src -/- and heterozygous c-src -/+ mice and their wild-type (c-src +/+) littermates. In the case of c-src +/+ mice, TCDD caused a decrease in body weight, an increase in the weight of liver, and significant reductions

Table 1. Effect of 115 μg/kg single i.p. TCDD dosing on organ and body weights of c-src -/+, and +/+ mice and geldanamycin-treated and nontreated +/+ mice

Strain	treatment	Initial Body weights (g)	Body weight (g)	Liver (g)	(% of Body weight)	Adipose tissue (g)	Thymus (g)
Experiment I							
+/+	Control	20.6 ± 1.3	23.5 ± 1.7	1.03 ± 0.10	(4.39 ± 0.43)	0.642 ± 0.064	0.056 ± 0.005
	TCDD	21.6 ± 2.3	18.1 ± 1.0**	1.06 ± 0.16*	(5.86 ± 0.88**)	0.308 ± 0.087**	0.023 ± .006**
-/+	Control	22.8 ± 2.5	28.4 ± 1.9	1.26 ± 0.20	(4.43 ±0.70)	0.656 ± 0.069	0.045 ± 0.004
	TCDD	24.3 ± 1.9	23.7 ± 3.1	1.70 ± 0.18**	(7.15 ± 0.76**)	0.642 ± 0.157	0.040 ± 0.004
Experiment II							
+/+	Control	26.8 ± 1.0	28.3 ± 1.6	1.35 ± 0.22	(4.77 ± 0.78)	0.886 ± 0.139	0.049 ± 0.003
	TCDD	28.2 ± 1.3	26.0 ± 0.7	1.76 ± 0.13*	(6.77 ± 0.50**)	0.478 ± 0.063**	0.019 ± .003**
+/+	Geldanamycin	25.8 ± 1.5	24.7 ± 0.5	1.27 ± 0.14	(5.14 ± 0.57)	0.774 ± 0.103	0.003 ± 0.003
	Geldanamycin + TCDD	26.6 ± 1.0	25.5 ± 1.1	1.64 ± 0.15*	(6.43 ± 0.59**)	0.632 ± 0.107	0.004 ± 0.002

*,**Statistically different from corresponding control values at P≤0.05 and 0.01, respectively (according to the Cochran *t*-test). Values are means ± SD; five mice were used for each group.

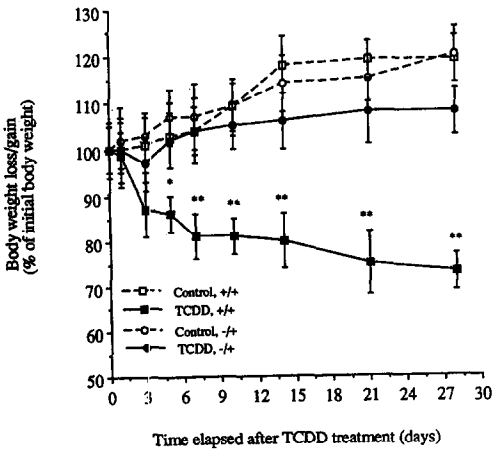

Fig. 1. Daily recording of body weights of c-src +/+ and -/+ mice treated with 115 μg/kg of TCDD. The data are expressed as % of the initial body weight of each strain. Controls were treated with vehicle only. The absolute values for the initial body weight are identical to those shown in Table 1, Experiment I.
*, ** Statistically different from corresponding control values at each time point at P≤0.05 and 0.01, respectively.

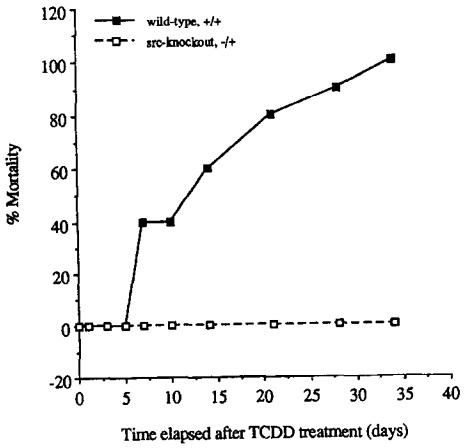

Fig. 2. Time course of lethal effect of high dose TCDD (345 μg/kg, single i.p. treatment) on wild-type littermate (+/+) mice and on heterozygous deficient (-/+) mice.

in the weight of adipose tissue and thymus (Table 1) and modest decreases in the weight of spleen, pancreas, and heart (data not shown), as expected. In contrast, the effects of the same TCDD treatment on changes in the weight of the body, adipose tissue and thymus were not significant in c-src -/+ mice. Because of the scarcity of the homozygous c-src deficient mice (c-src -/-) only one animal each, one for control and one for TCDD treatment at 115 μg/kg i.p. was tested. The result (data not shown) showed that the trend was similar in c-src -/- mice. On the other hand, the effect of TCDD to cause hepatomegaly (increased liver weight) was observed in all three strains. When the data were expressed in terms of the percentage of liver weight to body weight, this phenomenon of hepatomegaly became much more noticeable. To ascertain the responsiveness of the wild-type, c-src +/+ mice to TCDD, hepatic microsomal preparations from control and TCDD-treated (115 μg/kg after 10 days) mice were tested for EROD (7-ethoxyresorufin-0-deethylase) activities [16]. As expected EROD activity was found to be induced in TCDD-treated mice, actual values being 500 ± 51 (5 animals) for control and 1300 ± 115 pmol/mg proteins (5 animals) for TCDD-treated. When the same test was conducted on the c-src -/+ mice, almost the same degree of induction was observed, the actual values being 510 ± 40 (5) and 1500 ± 120 (5) pmol/mg protein for control and TCDD-treated -/+ mice, respectively.

To investigate whether the above phenomenon of reduced TCDD toxicity in c-src deficient mice is due to reduced c-Src kinase activities, the c-src +/+ mice were treated with geldanamycin, a tyrosine kinase inhibitor known to specifically prevent activation of c-Src kinase or its very closely related kinases [17], and the same TCDD toxicity tests were repeated. The results (Experiment II, Table 1) clearly showed that this chemical blocking method on c-Src kinase also produced the same trend with respect to the reduction of the body weight and adipose tissue loss as the genetic manipulation to suppress c-src expression. On the other hand, geldanamycin itself showed the effect to induce thymic atrophy. Therefore, no conclusion should be made on the effect of TCDD on this organ. The results shown in Fig. 1 also indicate the same trend, i.e. the reduction in the *in vivo* toxicity of TCDD in c-src deficient mice was also manifested in terms of daily body weight changes, where the effect of TCDD was clearly observed in c-src +/+ but not in -/+ mice.

To confirm that the above reduction in the effect of TCDD on organ weights of c-src deficient mice is related to reduction in the *in vivo* toxicity of TCDD, a higher dose of TCDD (345 μg/kg, which is known to cause 100% lethality in C57/Black mice) was tested on c-src -/+ and +/+ mice. As a result of this treatment, all of the c-src +/+ mice died (the last one died on day 34) as expected (Fig. 2), but none of the c-src -/+ mice died during the test period. This experiment is still in progress and as of the time of the re-submission of this manuscript (2/8/97, i.e. 230 days post-treatment) all c-src -/+ mice are still alive, gaining weight and showing no visible or overt signs of TCDD toxicity.

Since hepatomegaly was a clear sign of TCDD toxicity in all strains of mice tested, unlike any other criteria studied thus far, we examined the effects of TCDD on histological changes in their livers in an effort to understand the nature of the changes taking place in this tissue. Histological examination of liver sections showed a marked difference between the samples from untreated c-src +/+, wild-type control (Fig. 3A) and wild-type mice exposed to 115 μg/kg TCDD (Fig. 3B). The TCDD-treated mouse liver showed a panlobular lipid accumulation in cytoplasmic vacuoles within the hepatocytes. The presence of vacuoles throughout the liver parenchyma was apparent in both methylene blue-basic fuchsin and PAS-stained sections. TCDD-treated liver had a marked decrease in glycogen compared with control levels as confirmed by the PAS-stained tissue sections. There was also a large amount of inflammation and some ballooning degeneration and necrosis in the TCDD-

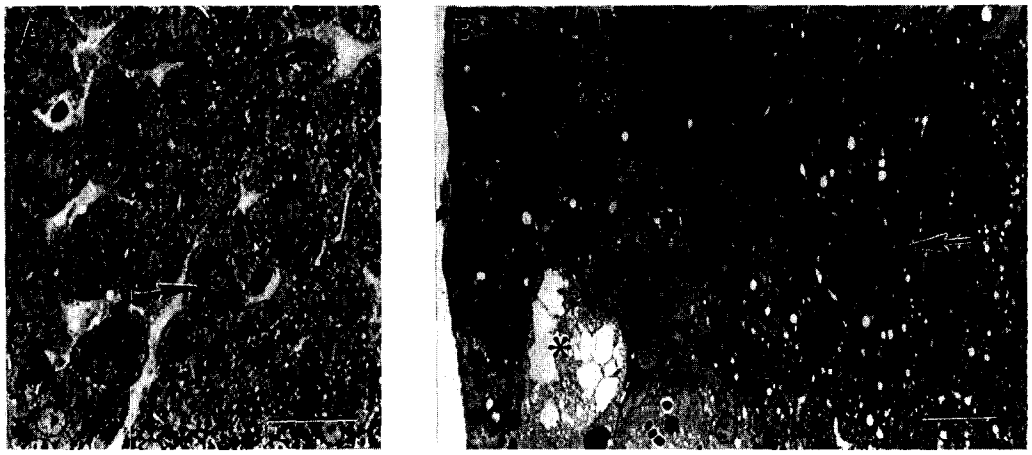

Fig. 3. PAS-stained liver sections from c-src +/+ wild-type mice: (A) control (note abundant glycogen granules shown by arrow) within hepatocytes; (B) TCDD treated. Note the necrosis (N), ballooning degeneration (*), and decrease in glycogen (arrow). Scale bars = 25 μm.

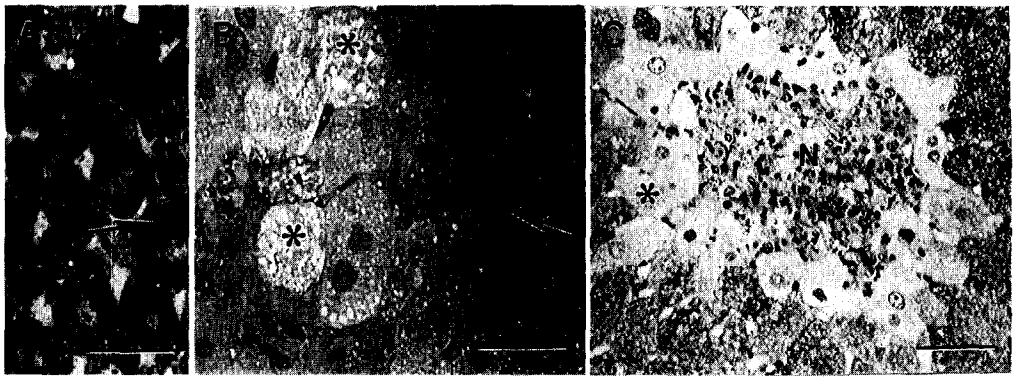

Fig. 4. PAS-stained liver sections from c-src -/+ mice: (A) control, (B) and (C) TCDD treated. Designations and scale bars are identical to those of Fig. 3.

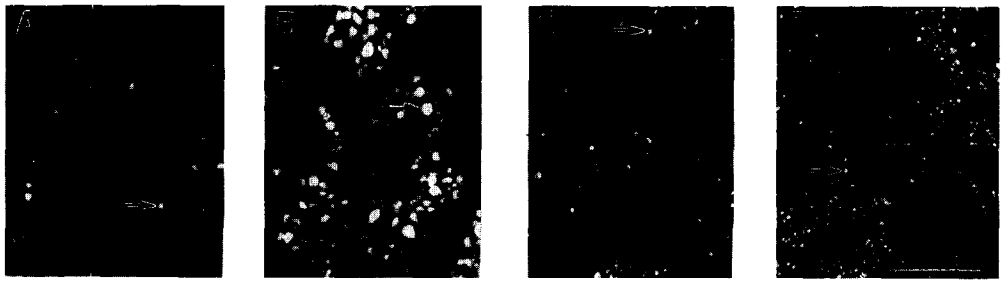

Fig. 5. Nile red-stained liver sections: (A) c-src +/+ control (note white lipid droplets shown by arrow), (B) +/+ TCDD treated, (C) c-src -/+ control, and (D) -/+ TCDD treated. Scale bar = 25 μm.

treated liver (Fig. 3B).

The 115 μg/kg TCDD-treated c-src -/+ mouse liver showed a different pattern of injuries (Fig. 4, A-C) as compared with that of the wild-type mouse. There was some cytoplasmic lipid accumulation over control levels (Fig. 5, C and D), but not nearly as much as in the samples from wild-type TCDD-treated mice (Fig. 5, A and B). However, the samples from the TCDD-treated, c-src deficient mouse had several areas of moderate ballooning degeneration (Fig. 4B) and some evidence of necrosis (Fig. 4C), as seen in both methylene blue-basic fuchsin- and PAS-stained sections. The remaining normal hepatocytes had the same amounts of glycogen as the c-src deficient control (Fig. 4, A and B), unlike the wild-type, TCDD-treated hepatocytes which had a decrease in glycogen. The presence of lipids within these vacuoles was confirmed by Nile Red-stained tissue sections (Fig. 5, A and B). In the case of -/+ mice, the extent of TCDD-induced lipid droplet accumulation was less (Fig. 5, C and D).

In summary these histological study results indicated that even in the case of hepatomegaly, which occurred in both c-src deficient and in wild-type mice, the expression of certain characteristic effects of TCDD, such as excessive accumulation of fatty droplets and depletion of glycogen, were clearly reduced in c-src deficient mice.

DISCUSSION

In the current study we have shown that the overall levels of acute toxicity of TCDD in c-src deficient (i.e. c-src -/+ and -/-) mice were less severe than that found in c-src +/+ wild-type littermates. This phenomenon was expressed most clearly in the case of the lethality test (Fig. 2) conducted at a high dose of 345 μg/kg. The fact that no c-src -/+ mice died, as compared with 100% mortality observed in the +/+ wild-type mice, unambiguously demonstrates that the acute toxicity of TCDD is not fully expressed in the c-src deficient mice. It must be mentioned here that the above conclusion is limited to specific criteria of acute toxicities as defined in the current study, e.g. the lethality at a high dose, and the loss of weight in adipose tissue, thymus and the body. Certainly, it is entirely possible that some potentially serious acute and chronic effects are still taking place in c-src deficient mice and that we may have missed those signs in this study. On the other hand, while several toxic expressions were reduced in c-src deficient mice, the most noticeable exception was hepatomegaly, which was found to take place in all strains (Table 1). The results of histological examinations indicated that the lesions occurring in c-src deficient -/+ mice were qualitatively different from those observed in c-src +/+ mice (Figs. 3-5). In the latter strain, the predominant lesions were fatty deposits and the disappearance of glycogen granules, whereas in the former strain these two lesions were relatively minor (or in some cases nonexistent). Instead, the necrosis and the ballooning degeneration were the most visible signs of toxic effects of TCDD. Edema formation is one of the well documented toxic endpoints of TCDD, occurring in mouse liver as a result of the action of TCDD [1-3]. Therefore, it is not surprising that this lesion was observed in these mice. Rather, the surprise was the finding that this particular endpoint was not affected at all by c-src deficiency, while other well documented lesions, such as fatty deposits and glycogen reductions, were clearly reduced. Such an observation favors the view that the effect of c-src deficiency manifests itself very selectively on the development of some specific toxic endpoints of TCDD, while sparing others.

One of the most noticeable differences between c-src deficient and wild-type mice with respect to their response to TCDD appears to be the apparent lack of a typical "wasting syndrome" in the former group. This hallmark effect of TCDD involves the loss of body weight, particularly the weight of adipose tissue, and the depletion of glycogen, all of which were found to be less fully expressed in

c-src deficient mice. However, much more work would be needed to prove that all aspects of the wasting syndrome are affected by c-src deficiency.

As to the cause for the reduction of the toxicity of TCDD in c-src deficient mice, our current working hypothesis is that c-Src kinase plays a pivotal role in transducing the signal of TCDD, c-Src kinase being physically associated with the Ah receptor [14], and activated upon the arrival of TCDD or any other active ligand and thereby serving as an essential messenger in one of the toxic signal transduction pathways of TCDD [9, 11]. The question whether such a hypothesis would adequately explain the whole phenomenon of altered toxicity of TCDD in c-src deficient mice cannot be answered at this time, since this line of investigation is still at a very incipient stage. It is important to point out, therefore, that the findings presented in this report by themselves do not distinguish between the secondary modulating influence of c-Src kinase activity versus its causal involvement. Much more work would be needed to establish the mechanism of c-Src involvement in the toxic action of TCDD in the future. In conclusion, we could clearly show that certain types of the toxicity of TCDD are not fully expressed in c-src deficient mice. The toxic effects that were clearly reduced in c-src deficient mice are: (a) the weight of several diagnostic organs and the body, and (b) lethality. Even in the case of hepatomegaly, which did not appear to be affected by c-src deficiency by the criterion of organ weight change, two major liver lesions (i.e. excess fatty deposit and glycogen depletion) were not fully expressed in c-src deficient mice. Certainly it is true that these are still very crude assessments of the toxicity of TCDD and that much more work would be needed to elucidate the mechanism by which c-Src participates in mediating TCDD toxicity. Nevertheless, together these data offer exciting future research possibilities and approaches. Fortunately, c-src is one of the best studied proto-oncogenes [14, 15], offering an excellent opportunity and a solid tool for toxicologists to explore this research avenue in the future.

Acknowledgements - This work was supported by ES03575, ES05233, and ES05707 from the National Institute of Environmental Health Sciences. We thank Dr. H. Osada of Riken Institute, Saitama, Japan, for the generous gift of geldanamycin and for his advice concerning the specificity of its action.

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