Comparison of *in vivo* effects of TCDD between heterozygous (-/+) and homozygous (-/-) c-src knockout, B6, 129-Src^{tm1sor} mice and between geldanamycin-treated and nontreated C57BL/6J mice

Debra Y. Dunlap, Essam Enan, Zhong Wu and Fumio Matsumura

Department of Environmental Toxicology University of California,
One Shields Avenue
Davis, California 956165, USA

Previously we have shown that TCDD, when given under cell free conditions to isolated cytosol fraction from guinea pig adipocytes, causes a significant rise in protein tyrosine kinase activities within 10 minutes (1). Subsequent studies in this laboratory have shown that the kinase thus activated is c-Src of which activation is clearly mediated by the Ah receptor (2,3). Based on such a background, we have proposed a hypothesis that at least a part of TCDD-caused toxic effects is medicated by c-Src kinase (3). In this scheme, activated c-Src is proposed to trigger various cellular responses particularly those known to be directly activated by this kinase as well as the systems involved in the growth factor signal transduction pathway.

Recently a strain of c-src knockout mice, B6, 129-Src^{tm1sor} has become available to us. This strain was originally developed by Dr. P. Soriano's group (4). The initial *in vivo* tests on the effect of TCDD on the heterozygous from (i.e. c-src 1/+ mice) in comparison to wild-type +/+ mice showed some of the toxic effects of TCDD, such as the body weight loss, the reduction in the wet weight of adipose tissue and thymus appeared to be less conspicuously expressed in the former strain (5). However, more recently we discovered that several shipments of wild-type +/+ mice contained C57BL/6J mice in addition to +/+ littermates of B6, 129-Src^{tm1sor}-/+ strain. C57BL/6J is a naturally TCDD susceptible strain as compared to 129 strain and therefore, if such a control was used, the above comparison study would not be valid. In addition, since B6, 129 is not an inbred strain, still containing segregating genes between C57BL/6 and 129, it is expected that some individuals could be carrying phenotypically expressed TCDD-susceptible (C57 lineage) characteristics among randomly selected B6, 129-Src^{tm1sor} +/+ mice. Certainly the best solution would be to develop a c-Src deficient C57BL congenic, mice, but it takes many generations of back-crossing to obtain such a congenic strain. In this study, therefore, we have made two approaches to circumvent the above problem: one to compare the toxic effects of TCDD between B6, 129 c-src -/- (homozygous) mice and their carefully matched c-src -/+ counterparts selected in our own laboratory, and second to study the protective effect of geldanamycin, a specific Src kinase inhibitor, in C57BL/65 mice to determine the effect of chemical blocking of c-Src *in vivo* within this TCDD-susceptible strain of mice.

EXPERIMENTAL METHODS

The original strain of B6, 129-Src^{tm1sor} mice were obtained from Jackson Laboratory (Bar Harbor, ME) as certified heterozygous c-src -/+ individuals. Homozygous -/- individuals were obtained in our laboratory as the result of crossings between pairs of heterozygous males and females. Homozygous offsprings were recognized by the lack of incisor development and the late eye opening. C57BL/6J mice were obtained from Jackson Laboratory as well as locally from Simonsen Labs Inc (Gilroy, CA). In all cases, mice were

treated with a single dose of 115μg/kg of TCDD intraperitoneally and sacrificed on day 10 of post-treatment. Geldanamycin was given intraperitoneally at 300 μg/kg on day -1, 3 and 7, matching control receiving the same volume of vehicle DMSO and corn oil only. The methods employed to study the enzyme or receptor binding activities were from the following published sources[@]. EROD (6), aldehyde dehydrogenase (7), DT-diaphorase (18), UDP-glucuronyl transferase (19), binding of ^{3}H 17-β-estradiol (10)^{125}, I-epidermal growth factor (11) and 3H-cytochalasin B to their receptors (12) and lipid peroxidation reactions (13). All data are expressed as means ± standard deviation and statistical significance of differences between control and treated were assessed using Cochran-t- test and the levels of significance are shown at $p \leq 0.05$ (*) or $p \leq 0.01$ (**).

RESULTS AND DISCUSSION

The effects of *in vivo* TCDD administration (115 μg/kg, single i.p. injection) on the weights of body and selected organs were assessed at day 10 in c-src -/- mice and in their littermates, -/+ mice (Table 1). The main differences between these two strains are: (a) thymic atrophy which was clearly observable in -/+ was slightly less significant in -/- mice, and (b) the extent of hepatomegaly was more pronounced in -/- as compared to -/+ mice. On the other hand, the reduction in the weight of adipose tissue was not statistically significant in either strain. It was noticed that the most consistent sign of toxicity of TCDD at this treatment regimen was the changes in the external appearance of the affected liver. In most severe cases, the liver surface assumes "mottled" appearance including fatty deposits , mosaic of excess coloration and discoloration and bumpy surface textures. According to the severity of the symptom, we have developed a scoring system: i.e. giving score of **5** for "mottled" stage, **4** for "dimpled" appearance, **3** for fatty spots on the surface, **2** for the surface dryness due to the insufficiency of peritoneal fluid, **1** for "light" appearance and **0** for normal appearance. By these criteria, the effect of TCDD was consistently observed in -/+ mice (rating of 2.27), but less in -/- mice (rating of 1.30).

To study the effect of c-src deficiency on the toxic action of TCDD at biochemical levels, we adopted 8 study parameters (two in adipose tissue and 6 in liver) all of which have been well documented to take place as the result of TCDD's action (Table 2). In the case of c-src -/- mice, induction of 3 out or 4 detoxification enzymes took place normally as compared to the case of c-src -/+ mice. Also the extent of increased membrane lipid peroxidation in adipose tissue was similar in these two strains. On the other hand, there were no statistically significant effects of TCDD on the levels of ligand binding to the EGF or the cytochalsin B receptor. Surprisingly ^{3}H-17 β -estradiol binding was upregulated in both strains instead of downregulated (10) as a result of TCDD's action. The same biochemical experiment was repeated in TCDD-treated regular and geldanamycin co-treated C57 BL/6J mice (Table 3). The results clearly indicated that the co-treatment with geldanamycin reduced the effects of TCDD on the EGF, the cytochalasin B and estrogen receptor, but had no effect on any other parameters.

The selection of well matched wild-type control for any hybrid type strain of knockout mice is not easy. This problem is particularly serious in the case of B6, 129-src^{tm1sor} mice, since B6 (=C57BL/6J), and 129 strain of mice show a significant difference in their sensitivity to TCDD. In any of given population of B6, 129 mice, it is expected that the ratio of B6/B6, B6/219, and 129/129 individuals is 1:2:1. Therefore, it is necessary to test a large enough population for each test group to avoid inappropriate comparison of phenotypically B6 dominating population vs 129 dominating ones. In the current study , the minimum of 5 animals per group was utilized to make the comparison in Table 1 experiment. The probability of all of 5 animals consisting of either B6/B6 or 129/129 is $(1/4)^{5}$. Under this circumstance, the rating of the liver appearance gave the consistent difference among src +/+, -/+ and -/- strains. In contrast, geldanamycin co-treatment study was done on the same strain (C57) as the control mice. Therefore, in this case the question on the genetic difference does not apply. On the other hand, although this compound is probably the most specific inhibitor available for c-Src kinase, one cannot totally exclude the possibility that other systems than c-Src kinase are affected by this co-treatment. Therefore, these limitations must be clearly understood in interpreting our current study results.

In conclusion, the most consistent trend we have observed in this study is that c-Src deficiency rescues the mice to develop down regulation of the EGF receptor and estrogen receptor and glucose transporters (=cytochalasin B receptor). On the organ level, c-Src deficiency appears to protect mice to develop severe case of fatty liver.

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Table 1. Effect of 115μg/kg single i.p. dosing of TCDD on organ weights of male B6, 129; Src^{tm1sor} -/+and -/- mice.

Tissues (mg wet weight)	B6, 129-Src^{tm1sor} males			
	-/+		-/-	
	Control	*TCDD*	*Control*	*TCDD*
Liver	1,350 ± 83 (6)	1,949 ± 157 (13)[a]	918 ± 84 (5)	1,540 ± 253 (5)[b]
Adipose Tissue	481 ± 150 (6)	398 ± 110 (13)	381 ± 169 (5)	332 ± 105 (5)
Thymus	27.6 ± 3.7 (6)	11.4 ± 9.6 (14)	27.8 ± 7.1 (5)	13.7 ± 13.2 (5)
Spleen	77.4 ± 12.6 (6)	95.3 ± 29.4 (8)	83.5 ± 28.1(4)	101.8± 25.9 (5)

[a]Liver toxicity appearance rating of 2.27 for 13 samples.
[b]Rating of 1.30 for 5 samples. All control groups showed "0" rating. For reference another TCDD-treated strain of B6, 129 +/+ mice (Fos-^{tm1sor}) showed 4.20 rating for 5 samples under the same treatment condition.

Table 2. Comparison of selected biochemical effects of TCDD between male B5, 129-Src^{tm1sor} -/+ and -/- mice (14).

	B6, 129-Src^{tm1sor} male mice			
	-/+		-/-	
Biochemical Parameters[a]	Control	TCDD	Control	TCDD
Liver				
EROD	510 ± 40 (5)	1500 ± 129**(5)	499 ± 35(3)	1650 ± 99**(3)
ADH Class 3	2.40 ± 0.20(3)	4.00 ± 0.60*(3)	2.00 ± 0.08(3)	5.00 ± 0.50**(3)
DT-diaphorase	0.51 ± 0.01(3)	1.01 ± 0.08*(3)	0.54 ± 0.02(3)	1.23 ± 0.10**(3)
UGT	3.31 ± 0.11(3)	5.63 ± 0.41**(3)	4.909 ± 0.32(3)	3.81 ± 0.34(3)
^{125}I-EGF binding	14.0 ± 0.5(5)	11.5 ± 0.8(5)	10.7 ± 0.6(5)	9.7 ± 0.3(3)
^{3}H-E2 binding	7.94 ± 0.77(5)	9.93 ± 0.74	4.46 ± 0.13(3)	8.31 ± 0.65**(3)
Adipose Tissue				
Lipid peroxidation	2.75 ± 0.90(3)	4.40 ± 0.80*(3)	2.90 ± 0.70(3)	4.60 ± 0.90*(3)
^{3}H-cytochalasin B	121 ± 10(5)	118 ± 9(3)	125 ± 11(5)	125 ± 8(3)

[a]Units used for these parameters are (top to bottom): pmol/mg/min, nmoles/min/mg, μmoles/min/mg, nmoles/min/mg, pg/400μg protein, pg/mg/protein, nmoles/mg protein and pmoles/mg, respectively.

Table 3. Comparison of selected biochemical effects of TCDD between male C57Bl/6J mice co-treated or untreated with 300 μg/kg of geldanamycin, a specific inhibitor of Src type tyrosine kinases (14).

	C57 Bl/6J male mice			
	Untreated		geldanamycin-treated	
	Control	TCDD	Control	TCDD
Liver				
EROD	600 ± 51(5)	1300 ± 115**(5)	440 ± 38(5)	150 ± 101**(5)
ADH	2.60 ± 0.10(3)	3.70 ± 0.09*(3)	2.50 ± 0.02(3)	3.60 ± 0.10*(3)
DT-diaphorase	0.39 ± 0.01(3)	0.70 ± 0.02**(3)	0.42 ± 0.06(3)	0.83 ± 0.07**(3)
UGT	1.83 ± 0.09(3)	2.56 ± 0.19**(3)	2.44 ± 0.22(3)	3.76 ± 0.11**(3)
^{125}I-EGF binding	17.7 ± 0.9(5)	2.45 ± 0.1**(5)	15.9 ± 0.7(5)	12.7 ± 0.4(5)
^{3}H-E2 binding	8.76 ± 0.90(5)	4.67 ± 0.11**(5)	9.19 ± 0.36(5)	9.37 ± 0.78(5)
Adipose tissue				
Lipid peroxidation	1.18 ± 0.1(3)	2.09 ± 0.13*(5)	1.09 ± 0.08(3)	1.96 ± 0.10*(3)
^{3}H-cytochalasin B	128 ± 9(5)	70 ± 5*(5)	110 ± 9(5)	105 ± 8(5)

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