Differences in binding of epidermal growth factor to liver membranes of TCDD-resistant and TCDD-sensitive rats after a single dose of TCDD

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TCDD (2,3,7,8-tetrachlorodibenzo-p-dioxin) has been demonstrated to decrease the binding capacity of epidermal growth factor (EGF) receptor.[1] This effect has been ascribed to be Ah locus dependent,[2] and hypothesized to be involved in several effects of TCDD such as toxicity in neonatal mice, effects on differentiation of keratinocytes, ureteric cell proliferation, palatal development in mouse embryos, and hepatocarcinogenic actions.
A large body of the data explaining the mechanisms of the actions of TCDD, exploit data on Ah locus in two strains of mice, C5BL/6J and DBA/2, which show a sensitivity difference of one order of magnitude. Also congenic strains assumed to differ only at the Ah locus have been produced by cross-breeding.[3] These data generally support the notion that most effects of TCDD are mediated through Ah receptor. In rats, however, two rat substrains, Han/Wistar (Kuopio, H/W) and Long-Evans (Turku AB, L-E) exhibit a sensitivity difference of three orders of magnitude.[4,5] Yet all measured parameters involving Ah receptors or AHH induction are identical in these strains.[6] This questions the validity of the Ah hypothesis in explaining all aspects of toxicity and its species and strain variation. Therefore this study was undertaken to examine the correlations of the toxicity of TCDD and EGF receptor changes in these two rat strains.

H/W and L-E male rats were housed in stainless steel wire-mesh cages before use at the age of 16-18 (H/W) and 19-21 (L-E) weeks. They were fed ad lib. with pelleted R3 rat feed (Ewos, Södertälje, Sweden) and tap water.
TCDD was dissolved to corn oil and given i.p. H/W rats were treated with doses of 50 and 500 μg/kg, both of which are non-lethal in this strain, and L-E rats with 5 μg/kg which is sublethal, and with 50 μg/kg which is usually 100% lethal to this strain. Controls were given the equivalent volume of corn oil.
After 4 or 10 days the rats were anesthetized with carbon dioxide + oxygen, and livers

were dissected out and stored frozen in -80°C until membrane preparations were made within 7 weeks. Liver samples (1.5-2 g) were homogenized in loosely fitting Dounce glass homogenizers in the presence of protease inhibitors (leupeptin and phenylmethylsulfonyl fluoride) and membranes isolated by differential and Percoll™ density gradient centrifugation as previously described.[2,7] The protein concentration was adjusted to 0.8 mg/ml, and 50 µl aliquots were incubated in the total volume of 200 µl buffer pH 7.4 with various concentrations of ^{125}I-labeled EGF. After 1 hour incubation at room temperature the membranes were separated by glass fiber filters over vacuum, and the ligand bound to the filters was counted in gamma counter. Nonspecific binding was determined in the presence of excess cold EGF (200 nM). EGF, Mouse, Natural, Culture Grade was from the Collaborative Research Inc., Bedford, Mass., and it was custom labeled with ^{125}I by Diagnostic Systems Laboratories, Inc., Webster, TX. Iodinated EGF (59.09 µCi/µg, nominal concentration of EGF 5 µg/2 ml giving 8.8 nM incubation concentration) was diluted with 5 nM cold EGF to adjust the concentrations to provide assay points on both sides of the K_D, and the resulting nominal concentration of 13.8 nM EGF was diluted by serial dilution for binding studies.

ReceptorFit Saturation Two-site (Lundon Software, Inc., Chagrin Falls, OH) was used to calculate the kinetic constants of the receptor binding. SPSS/PC+ was used for statistical treatment of the data. Analysis of variance (ANOVA) was used for statistical assessment of the data, followed by Duncan's post hoc test in assessing the significance of difference between two groups. In some cases t-test was utilized. The data are shown as means ± SD.

Table 1. Weights and liver weights of rats in different dosage groups. Means ± SD were determined, the number of experiments is given in parentheses. Asterisks indicate statistically significant difference from the respective control (One-way ANOVA, * $p<0.05$).

Strain and treatment	Exposure 4 Days Body weight g initial	final	Liver weight g	Exposure 10 Days Body weight g initial	final	Liver weight g
H/W Control (6)	380±43	384±40	13.2±1.6	369±39	380±42	12.8±1.4
H/W 50 µg/kg (6)	379±26	385±28	16.7±2.5*	383±30	375±22	17.4±1.7*
H/W 500 µg/kg (5)	380±41	389±39	18.8±2.9*	377±28	355±27	15.2±1.7*
L-E Control (6)	324±16	331±17	12.9±1.1	329±23	337±24	13.2±1.3
L-E 5 µg/kg (6)	n.d.	n.d.	n.d.	330±14	319±18	15.5±1.8
L-E 50 µg/kg (6,7)	334±12	315±7	14.5±0.8*	337±25	282±14*	9.6±1.6*

n.d. = not determined

The effect of TCDD on the weight of animals, as well as liver weights, are shown in Table 1. There were minimal effects on weight at either time point in H/W rats, but a clear reduction in L-E. Liver weight increased in H/W at both time points and in L-E at 4 days. However, at ten days L-E (50 μg/kg) exhibited a clear liver atrophy.
No differences were found in control K_D values between the strains (H/W 3.8±1.8 nM, L-E 3.9±1.6 nM, SD, n=12). B_{max} of L-E tended to be slightly higher (741±209 vs. 586±235 fmol/mg protein, n.s., t-test).
The kinetic constants of various groups are shown in Table 2. Observed changes in the K_D values were in both directions, and it remains to be seen if these are chance effects or related to increases/decreases in liver weight. On the other hand, both treatment and time effects on B_{max} were significant in ANOVA in both strains (HW: $p<0.01$; L-E: $p<0.001$). Three-way interaction was significant ($p<0.05$, strain, time and treatment 0 or 50μg/kg), so the strains responded to TCDD differently with time. Among individual groups there were significant differences only at 10 days (Table 2).

Table 2. Kinetic constants of EGF receptor binding. Rat liver membranes were incubated with various concentrations of ^{125}I-labeled EGF for 1 hour, and separated by membrane filtration. Kinetic constants were assessed and their means ± SD determined, the number of experiments is given in parentheses. Asterisks indicate statistically significant difference from the respective control (t-test, * $p<0.05$, *** $p<0.001$).

Strain and treatment	Exposure 4 Days K_D nM	Exposure 4 Days B_{max} fmol/mg	Exposure 10 Days K_D nM	Exposure 10 Days B_{max} fmol/mg
H/W Control (6)	5.30±1.02	712±257	2.32±0.95	459±132
H/W 50 μg/kg (6)	6.05±0.86	492±147	2.30±0.94	319±225
H/W 500 μg/kg (5)	6.77±2.39	432±164	4.44±1.93	254±33*
L-E Control (6)	4.51±1.78	801±217	3.23±1.13	680±201
L-E 5 μg/kg (6)	n.d.	n.d.	5.42±1.87*	632±107
L-E 50 μg/kg (6,7)	7.77±4.26	648±220	1.67±0.44*	144±68***

n.d. = not determined

Hence a quantitative difference in EGF receptor binding characteristics by liver membranes was seen between the TCDD-resistant H/W rat and the TCDD-sensitive L-E rat strain. This is a most interesting finding, because most responses to TCDD save wasting and lethality are similar in these two strains. The crucial question is whether this difference is primary and related to the mechanism of difference in susceptibility between these strains, or if it is secondary and perhaps related to other, primary changes due to TCDD or even to liver atrophy and wasting which are well

underway in L-E rats by day 10 after the exposure.
It appears that the differences in the downregulaton of EGF receptor do not correlate well with the large strain difference in lethality. Neither do they correlate with the liver Ah-receptor binding and enzyme induction which are identical in the two rat strains, and induction is observed around the doses of 1 μg/kg in both strains.[6]
In keratinocytes the proposed mechanism of EGF receptor downregulation by TCDD is overexpression of TGF_α and the consequent enhanced occupation by TGF_α and phosphorylation and internalization of the EGF receptor.[8] Whether or not the same is true with liver is not known, but tyrosine-specific protein phosphorylation[9] is consistent with this model. Overproduction of TGF_α could also play a crucial role in the tumor-promoting actions of TCDD. It remains to be seen, to what extent the difference in these rat strains can be used for clarifying the mechanisms and consequences of EGF receptor response.

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