EVIDENCE FOR AN INDIRECT MECHANISM OF ACUTE TOXICITY OF 2,3,7,8-TETRACHLORODIBENZO-p-DIOXIN IN RATS

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ABSTRACT

The major, if not sole, cause of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)-induced death in laboratory animals studied is a gradually increasing voluntary feed refusal. Much higher concentrations of TCDD were produced in various brain regions of rats including the hypothalamus after intracerebroventricular (i.c.v.) than after lethal intravenous (i.v.) injections; yet i.c.v. dosed rats thrived, whereas rats injected i.v. displayed the typical cachectic syndrome. These findings preclude the possibility of a direct effect of TCDD on central appetite regulation. It was further determined that TCDD-induced appetite suppression is of peripheral origin beginning with inhibition of phosphoenolpyruvate carboxykinase (PEPCK). The resulting reduction in gluconeogenesis leads to progressive increases of plasma tryptophan levels, which in turn appear to cause a serotonin-mediated reduction of feed intake. It is suggested that subchronic and chronic toxicities of TCDD and of related compounds may also be related to inhibition of key enzymes of gluconeogenesis.

MATERIALS, METHODS

Male Sprague-Dawley rats (225-275 gr: Sasco, Omaha, NE) were used in all experiments. They were allowed a 3 day acclimation period and were housed individually in a climate-controlled animal facility at 21±2°C and a 12 hr light/dark cycle (06:00 to 18:00). Animals had free access to tap water, and were fed Purina Rodent Chow 5001 (Ralston Purina, St. Louis, MO). All treatments were performed with TCDD (99% chemical purity, Cambridge Isotope Labs., Woburn, MA), or ^{3}H-TCDD (purity > 97%, specific activity 40 Ci/mmol: Cambridge Isotope Labs.) dissolved either in corn oil/acetone 95/5 (v/v), or in a 10% corn oil/water emulsion. Corn oil/acetone was administered i.p. at 4 ml/kg, the oil/water emulsion i.v. into the tail vein at 4 ml/kg.

Experiment 1: TCDD in corn oil/acetone was given i.c.v. at 8 μg/kg (80 μl/kg) with a standard stereotactic procedure into the right lateral ventricle. Intravenous TCDD in the oil/water emulsion was given at 72 μg/kg. Feed intake and body weights were recorded daily. Groups of 4 rats each were sacrificed at 1, 4, 8, and 32 days after treatment. Radioactivity in liver and various brain areas was determined by liquid scintillation counting, and TCDD concentrations calculated as ppb.

Experiment 2: Rats were treated i.p. with 5, 15, 25, 50, and 125 μg/kg TCDD. Body weight-matched control animals received vehicle only and were pair-fed to their TCDD-treated counterparts with one day's delay. Cumulative feed intake was calculated for days 4 through 7 after treatment; groups of 5 rats were sacrificed after an overnight fast on day 8 after treatment. Livers were removed and phosphoenolpyruvate carboxykinase (PEPCK) activity determined according to Petrescu *et al.* [1] in a 100,000g supernatant of liver homogenate, using deoxyguanosine 5'-diphosphate as the nucleotide substrate (this specifically avoids interference from pyruvate kinase). Oxaloacetate formed during the (reverse) enzyme reaction was determined spectrophotometrically at 340 nm with malate dehydrogenase in the presence of NADH. Reaction blanks contained neither bicarbonate nor CO_2.

Experiment 3: Rats received 125 μg/kg TCDD i.p., or vehicle alone. Vehicle-treated controls were pair-fed to their TCDD-treated counterparts. At 4, 8, and 16 days after dosing, groups of 5 animals received i.v. a 0.2 mM solution of L-alanine in saline (1 ml/kg), containing 50 μCi ^{14}C-alanine (specific activity 135 mCi/mmol: ICN Radiochemicals, Irvine, CA), and were sacrificed 10 min later. Gluconeogenesis was measured in deproteinized blood samples by determination of ^{14}C-alanine with ion exchange chromatography on Dowex 50Wx8 [2], and of ^{14}C-glucose with a batch

ion exchange procedure on Dowex 50Wx8 plus Duolite A-4 [3]. Concentrations of alanine and glucose in plasma were determined by standard enzymatic procedures. Results in TCDD-treated animals were expressed as percent of pair-fed controls.

Experiment 4: One group of rats received i.p. 125 µg/kg TCDD, two groups received vehicle only. One of the vehicle-treated groups was pair-fed to the TCDD-treated animals, the other group had ad libitum access to feed. Groups of 5 TCDD-treated and pair-fed animals, respectively, were decapitated on days 4, 8, and 16 after treatment, ad libitum-fed animals on day 8 only; trunk blood was collected in heparinized tubes and plasma prepared. Brains were also removed and dissected into several areas. Tryptophan was determined in extracts of plasma and hypothalamus and determined by HPLC on a reverse phase C18 column with fluorometric detection [4]. Serotonin (5-HT) and 5-hydroxy-indoleacetic acid (5-HIAA) in hypothalamus extracts were also determined by HPLC with fluorometric detection [5]. Statistical analyses were performed with Student's t-test.

RESULTS AND DISCUSSION

The acute toxicity of TCDD and related chlorinated hydrocarbons is characterized by distinct symptoms in essentially all mammalian species studied: progressive reduction of feed intake accompanied by body weight loss eventually resulting in death of the animals [6,7]. Initially, investigations focussed on altered body weight set-points of TCDD-treated rats [8,9]. However, as Fig. 1 shows, TCDD does not exert a direct effect on central appetite regulation. Injecting TCDD into the lateral cerebral ventricle produced concentrations in various regions of the brain much higher than a lethal i.v. dose without any adverse effect on feed intake or body weight [10], whereas the i.v. dose greatly impaired both (Fig. 1). This finding precludes the possibility of a direct effect of TCDD on the hypothalamus and suggests that the appetite suppressive effect of TCDD is of peripheral origin.

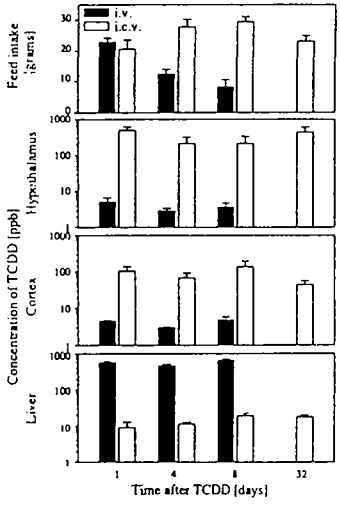

FIGURE 1: Feed intake (top panel), and concentrations of TCDD in two brain regions (middle panels) and liver (bottom panel) of rats after a non-lethal i.c.v. or a lethal i.v. injection

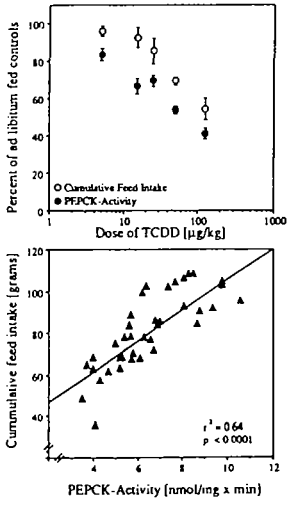

FIGURE 2: Dose-response of cumulative feed intake and PEPCK activity in TCDD-treated male rats (upper panel), and correlation between feed intake and PEPCK activity (lower panel)

It has been demonstrated that TCDD and other chlorinated hydrocarbons inhibit activities of key enzymes of gluconeogenesis like phosphoenolpyruvate carboxykinase, pyruvate carboxylase or glucose-6-phosphatase [11,12,13]. The earliest observed effect of TCDD is decreased activity of PEPCK one to two days after dosing [13]. PEPCK is considered one of the key enzymes regulating gluconeogenesis in long-term feed deprivation [14]. PEPCK responds to feed deprivation with increased activity [13]. However, in contrast to feed-deprived rats, PEPCK activity is decreased by TCDD in a dose-dependent fashion (Fig. 2). Moreover, the dose responses to inhibition of PEPCK activity and reduction of feed intake

occur in the same dose range (Fig. 2, upper panel) with a highly significant correlation (Fig. 2, lower panel). It is noteworthy that among the numerous biological effects of TCDD (e.g. enzyme induction, immunosuppression, chloracne, etc.), only gluconeogenic enzyme activities show dose response in the same range where appetite suppression occurs. The apparent consequence of decreased PEPCK activity is inhibition of gluconeogenesis by TCDD, demonstrated by decreased conversion of ^{14}C-alanine into ^{14}C-glucose (Table 1). As a likely sequel of its decreased utilization for gluconeogenesis, plasma alanine levels (both ^{14}C- and total alanine) progressively increase in TCDD-treated rats [15], together with some other glucogenic amino acids [16] including tryptophan (Fig. 3, upper panel). The role of tryptophan in 5-HT-mediated appetite suppression is well established [17,18] and accordingly its levels increase in the hypothalamus (Fig. 3, lower panel). As a consequence [17] 5-HT and more clearly 5-HIAA also increase in the hypothalamus of TCDD-treated rats (Fig. 4). It is important to point out that the time course of appetite suppression in TCDD-treated rats coincides with increased plasma and hypothalamic concentrations of tryptophan (Fig. 3) as well as with elevated 5-HT and more clearly with 5-HIAA levels in the hypothalamus (Fig. 4). Norepinephrine, dopamine and their metabolites are unaffected in the hypothalamus of TCDD-treated rats [19].

TABLE 1: Gluconeogenesis in TCDD-treated rats relative to pair-fed controls

Day after dosing with TCDD[a]	Blood ^{14}C-glucose (% of control)
4	76 ± 10[b]
8	51 ± 8
16	35 ± 12

[a] 125 μg TCDD i.p.
[b] Mean ± S.E.M., n = 5
Modified from [15]

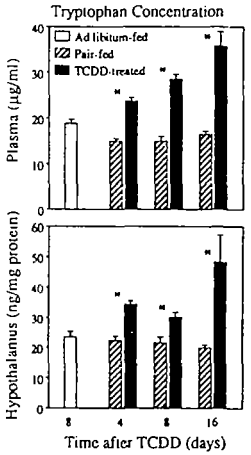

FIGURE 3: Time course of tryptophan concentrations in plasma (upper panel) and hypothalamus (lower panel) of TCDD-treated, pair-fed and ad libitum-fed rats after a lethal dose of 125 μg/kg i.p. * = signif. at $p < 0.05$.

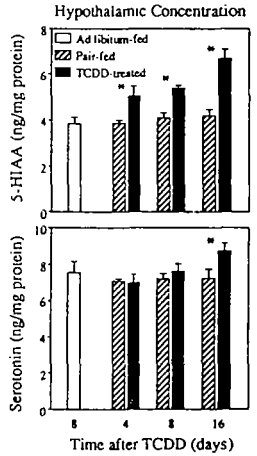

FIGURE 4: Time course of 5-HIAA (upper panel) and 5-HT (lower panel) concentrations in hypothalami of TCDD-treated, pair-fed and ad libitum-fed rats after a lethal dose of 125 μg/kg i.p. * = signif. at $p < 0.05$.

Thus, it is now possible to suggest a sequence of events constituting the acute toxicity of TCDD and most likely that of all toxic TCDD congeners in the rat. So far the earliest documented effect of TCDD is decreased activity of PEPCK by one to two days after dosing. This leads to inhibition of gluconeogenesis detected at four days after dosing. Inhibition of gluconeogenesis is the most likely cause of progressively increasing plasma tryptophan levels observed four to 16 days after dosing. This seems to result in a serotonergic appetite suppression, which appears to be the ultimate but indirect cause of the acute toxicity of TCDD. It needs to be emphasized that the likelihood of a coincidental correlation

for two events (inhibition of PEPCK activity and reduction of feed intake), separated by as many sequential steps as shown here, is quite small.

Clearly, the molecular basis of inhibition of PEPCK remains to be elucidated. However, there are far-reaching implications of this novel but certainly not unique mechanism of toxicity. Both the toxicity of TCDD and mechanisms of appetite regulation are highly species specific. Thus, TCDD and similar compounds may be suitable tools to study appetite regulation in various species with likely benefits for a better understanding of human appetite control mechanisms. Similarly, and perhaps more importantly, it is likely that many subchronic and chronic toxicities and possibly also carcinogenicity of TCDD and congeners are related to more subtle consequences of inhibition of gluconeogenic enzymes. Glucose-6-phosphatase has been used as marker enzyme for preneoplastic foci [20], although a cause-effect relationship has not been considered. However, Banasch *et al.* [21,22] pointed out a high correlation between glycogenosis (glycogen storage decrease) and related enzymatic changes (particularly glucose-6-phosphatase deficiency) and hepatocellular tumors. These authors suggested the possibility of a cause-effect relationship. TCDD causes both glycogenosis [16] and inhibition of glucose-6-phosphatase, as well as of pyruvate carboxylase activities [13], but PEPCK activity is affected either by lower doses, much earlier or both. Therefore, enzymes of gluconeogenesis, particularly PEPCK, and the molecular mechanisms of their regulation ought to be included in future studies of hepatocarcinogenesis.

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