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ALTERED REGULATION OF ADRENAL STEROIDOGENESIS IN 2,3,7,8-TETRACHLORODIBENZO-*p*-DIOXIN-TREATED RATS

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Abstract—A single treatment of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) (50 μg/kg) produced two distinct effects on adrenal steroidogenesis in rats 13 days post-treatment. In unstressed rats, the very low corticosterone levels early in the light phase (AM) increased 4-fold relative to *ad libitum*-fed control (ALC) rats, but the peak level of corticosterone that is seen late in the light phase (PM) decreased up to 40% relative to ALC rats. The AM stimulation was also observed in rats pair-fed to compensate for the diminished feed intake of TCDD-treated animals, indicating that the change results from nutritional deprivation. The PM suppression, however, was not observed in pair-fed rats. In rats given a lower dose of TCDD (15 μg/kg), there was no AM stimulation, whereas the suppression of the PM diurnal peak of corticosterone was retained. Plasma adrenocorticotropin (ACTH) levels and adrenal size were not changed by these treatments, indicating that TCDD affects adrenal responsiveness. TCDD did not, however, have a significant effect on corticosterone secretion in rats receiving high doses of ACTH. In control animals, the availability of cholesterol to cytochrome P-450$_{scc}$ limits the rate of steroidogenesis. While the specific content of the cytochrome was unaffected by TCDD, cholesterol turnover by this enzyme appeared to be affected following TCDD treatment, as evidenced by small increases in the mitochondrial levels of free cholesterol, reactive cholesterol, and in the proportion of P-450$_{scc}$ complexed with cholesterol relative to both *ad libitum*- and pair-fed controls. This accumulation of mitochondrial cholesterol following TCDD treatment is consistent with an inhibition of cholesterol metabolism at cytochrome P-450$_{scc}$ *in vivo* that is removed upon isolation of the mitochondria. These TCDD-induced increases were enhanced substantially in ACTH-stimulated rats, probably because ACTH enhances cholesterol influx into the mitochondria. Normally, substrate availability is rate limiting in cholesterol side-chain cleavage, and the AM stimulation of steroidogenesis by TCDD may result from such increased cholesterol transfer. The inhibition of cholesterol side-chain cleavage resulting from TCDD treatment may, however, only become rate limiting for corticosterone synthesis when cholesterol transfer is more substantially activated, as for peak PM secretion.

The toxicity of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD‖) in experimental animals is characterized by a general debilitation and progressive weight loss accompanied by decreased feed consumption and parallel mobilization of adipose tissue stores [1–6]. The regulation of nutritional balance and metabolic homeostasis in rats is evidently altered by TCDD exposure [7]. Other effects of TCDD, such as loss of muscle mass, hypoglycemia, hypercholesterolemia, suppression of the immune response, and the lack of toxicity in cultured cells [1, 2, 8] suggest that TCDD action may be mediated by changes in endocrine function. Previous investigations of the role of the adrenal gland in TCDD toxicity have suggested that glucocorticoids are not directly involved [9, 10]. Although glucocorticoids can induce thymic atrophy [10], adrenalectomy does not protect against TCDD-induced thymic atrophy, and physiologically elevated glucocorticoids alone do not mediate this effect [11]. Adrenalectomy, however, increases the lethal potency of TCDD in rats and decreases the latency period between TCDD administration and death [9]. This suggests that glucocorticoids may actually protect against TCDD toxicity.

A substantial rise in plasma corticosterone, after a lethal exposure of rats to TCDD, has been reported previously [9]. However, experimental details concerning the treatment of the rats (light cycle, stress, sampling time), that are shown in this study to be critical, were omitted. In this laboratory, a selective suppression of corticosterone secretion by TCDD has been observed late in the light cycle [12]. A second report in which TCDD suppressed plasma corticosterone levels [13] is difficult to interpret because of the unusually low control corticosterone levels (3 μg/dl) relative to those described here and elsewhere [14]. In this study, the diurnal dependence of the effect of TCDD on corticosterone secretion is examined.

TCDD treatment may affect serum corticosterone levels both through changes in pituitary adreno-

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‖ Abbreviations: TCDD, 2,3,7-8-tetrachlorodibenzo-*p*-dioxin; ACTH, adrenocorticotropin; SCC, side-chain cleavage; HPLC, high pressure liquid chromatography; PFC, pair-fed control; ALC, *ad libitum*-fed control; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; and cAMP, cyclic-3′,5′-adenosine monophosphate.

corticotropin (ACTH) secretion and from changes in the responsiveness of the adrenal cortex. The response of the adrenal cortex to ACTH is mediated by cyclic 3',5'-adenosine monophosphate (cAMP) and involves a stimulation of the rate-limiting step in steroidogenesis, the side-chain cleavage (SCC) of cholesterol to pregnenolone [15]. This step is catalyzed by a specific cytochrome P-450 ($P\text{-}450_{scc}$), located on the matrix side of the inner membrane of adrenal mitochondria. Acute ACTH exposure increases the availability of free cholesterol to adrenal mitochondrial $P\text{-}450_{scc}$ (side-chain cleavage P-450) for steroidogenesis [16–18]. Several steps are involved in this process that may be affected by TCDD: (a) uptake of cholesterol esters from plasma lipoproteins [19], (b) hydrolysis of cholesterol esters [20], (c) transport of cytosolic free cholesterol to mitochondria [21], and (d) intramitochondrial transfer of free cholesterol to $P\text{-}450_{scc}$ [16]. In rats, the diurnal rhythm of corticosterone secretion [14] is also paralleled by changes in the rate of adrenal mitochondrial cholesterol SCC activity [22].

The present study investigates changes in the acute regulation of steroidogenesis 13 days after single administrations of TCDD (4–50 μg/kg) by comparing results obtained from TCDD-treated rats with results obtained from control rats (both groups were fed *ad lib.* for 14–22 hr per day). The regulation of steroidogenesis in pair-fed control (PFC) rats was also investigated so that the extent to which the effects of TCDD were secondary to undernutrition could be estimated. The mechanisms underlying these changes in the adrenal cortex have been examined by measuring mitochondrial cholesterol levels and the quantity of cytochrome $P\text{-}450_{scc}$-cholesterol complex in isolated adrenal mitochondria. These variables provide sensitive indicators of changes in intracellular cholesterol movement.

MATERIALS AND METHODS

Chemicals. ACTH(1-24) (Cortosyn) was purchased from Organon, Inc. (West Orange, NJ). Cyanoketone (2α-cyano-4,4,17β-trimethyl-17α-hydroxy-5-androstene-3-one), an inhibitor of 3β-hydroxysteroid dehydrogenase, was donated by the Upjohn Co. (Kalamazoo, MI). Solvents for high pressure liquid chromatography (HPLC) and gas chromatography were purchased from Burdick & Jackson Laboratories, Inc. (Muskegon, MI). [7-^{3}H(n)]Pregnenolone and [1,2,6,7-^{3}H]corticosterone were obtained from New England Nuclear (Boston, MA); pregnenolone and corticosterone antibody were purchased from Radioassay Systems Laboratories, Inc. (Carson, CA) and Miles Laboratories (Elkhart, IN) respectively. All other chemicals and reagents, unless otherwise specified, were obtained from the Sigma Chemical Co. (St. Louis, MO).

Animal treatment. Male Sprague–Dawley rats (250–300 g) were obtained from Harlan Sprague–Dawley (Madison, WI) and housed in individual cages. Rats were maintained in a temperature-controlled room (20°) with a 12-hr light/dark cycle and were handled daily to minimize stress when the animals were killed. Ground rat chow (except pair-fed rats, 40 g/day, Ralston Purina, St. Louis, MO) was made available to rats under two different protocols, as designated in Fig. 1. (The original experiment was conducted using rats that had been on Schedule A for another purpose. Schedule B, the desired protocol, was used in subsequent experiments.) Water was available at all times. After a 3- to 6-day acclimation period, rats were treated (day 0) with either a single oral dose of TCDD (50 μg/kg, Dow Chemical Co., Midland, MI) or an equal volume of vehicle [acetone–corn oil (0.5:9.5, v/v), 1 ml/kg]. Body weight and feed consumption were recorded for each rat during the experimental period (usually 13 days). In the experiments that included pair-fed controls (PFC), rats were paired with weight-matched partners. PFC rats were allowed only the amount of feed (corrected for spillage) that their corresponding TCDD-treated partner consumed on the previous day [4]. Approximately 10–11 days following treatment, TCDD-treated rats exhibited feed consumption equivalent to only about one-half that of control rats fed *ad lib.* (ALC rats). Rats were killed by decapitation 20 min following an intraperitoneal injection of 2 units of ACTH (in 0.9% NaCl) or following conditions of no treatment (i.e. unstressed). Except where noted, all rats in a given experiment were killed at the same time, early (10:00 AM) or late (6:00 PM) in the light phase of the cycle. In a time–course experiment, unstressed rats were killed at several different times on day 13 post-treatment.

Subcellular fractionation. Adrenal glands were removed, decapsulated, and maintained in ice-cold phosphate-buffered saline (pH 7.4) until all adrenals were collected. Adrenal weights, when taken, were determined on defatted adrenals before decapsulation. Adrenal tissue was gently homogenized in 50 mM potassium phosphate containing 150 mM KCl and 0.1 mM EDTA (pH 7.4) at 4° in a Teflon–glass homogenizer and centrifuged at 600 *g* for 10 min. The supernatant fraction obtained from this step was centrifuged at 10,000 *g* for 15 min and the resulting mitochondrial pellet was washed by resuspension in homogenization buffer and recentrifuged at 10,000 *g* for 15 min. The post-mitochondrial supernatant fraction (10,000 *g* supernatant) was centrifuged for 90 min at 105,000 *g* and the resulting microsomal pellet was resuspended in 50 mM potassium phosphate buffer containing 20 mM Hepes, 0.1 mM dithiothreitol, 20% glycerol and 0.1 mM EDTA (pH 7.5). All spectral studies and enzyme activities were determined in fresh isolates on the day of preparation. Protein content of individual fractions was determined in trichloroacetic acid precipitates by the method of Lowry *et al.* [23] with bovine serum albumin as standard.

Spectral studies. Cytochrome P-450 content was determined in mitochondrial and microsomal fractions by the method of Omura and Sato [24]. Pregnenolone-induced reverse Type I and 20α,22*R*-dihydroxycholesterol-induced Type I spectral changes were measured in intact mitochondria as described previously [25]. Spectral changes in response to added steroids were measured in an Aminco DW-2 spectrophotometer, operating in the dual-wavelength mode while continuously recording

the difference in absorbance between 390 and 420 nm.

Enzyme assays. Mitochondrial cholesterol side-chain cleavage was measured in intact mitochondria (0.1 mg/ml protein in spectral assay buffer adjusted to pH 7.2 and containing 5 μM cyanoketone) as described previously using 25-hydroxycholesterol (30 μM, final concentration) as substrate. The addition of 25-hydroxycholesterol to mitochondrial suspensions allows for the measurement of cholesterol SCC activity when substrate availability is not limiting [26]. Pregnenolone formed in a 10-min incubation was quantitated by radioimmunoassay [27]. Steroid 11β-hydroxylation was measured as the production of corticosterone in 10 min in mitochondrial incubations containing 75 μM deoxycorticosterone and 10 mM isocitrate (in cholesterol SCC assay buffer) at 37°. Microsomal 21-hydroxylase activity was measured as the production in 10 min of deoxycorticosterone from progesterone (75 μM) at 37° in the presence of an NADPH-generating system (60 mM glucose-6-phosphate, 5 mM NADP, 3 mM $MgCl_2$, and 0.5 units/ml glucose-6-phosphate dehydrogenase, final concentrations) in 50 mM potassium phosphate buffer, pH 7.5. Corticosterone and deoxycorticosterone produced in these assays were determined from methylene chloride extracts by HPLC as described previously [28].

Plasma corticosterone and ACTH determinations. Several precautions to avoid stressing the animals were taken. Each rat was handled daily for at least 2 weeks, and personnel did not enter the animal quarters for several hours before rats were killed. Rats were killed in random or systematic sequences by decapitation (outside the animal quarters) within 30 sec after their cages were opened. Dosing with ACTH was not begun until all basal rats had been killed. Plasma for both ACTH and corticosterone measurements was prepared from blood collected with 1.5 mg/ml EDTA (final concentration) and centrifuged at 10,000 *g* for 15 min. Plasma for corticosterone determinations was prepared from heparinized blood (approximately 10 units/ml) by centrifugation at 1500 *g* for 15 min (4°). Plasma was separated from the pellet and stored at −20° until further use. Corticosterone was determined by radioimmunoassay [12], and ACTH was determined by the double antibody radioimmunoassay procedure using a kit purchased from the Immuno Nuclear Corp. (Stillwater, MN).

Cholesterol analysis. Cholesterol was extracted from intact mitochondria with methanol/ethyl acetate in the presence of 5α-cholestane as an internal standard [29]. Total mitochondrial cholesterol was quantified by gas chromatography as reported by Privalle *et al.* [16]. A pool of reactive mitochondrial cholesterol was assayed by carrying out side-chain cleavage as described above, except without addition of 25-hydroxycholesterol [17]. The metabolism of endogenous cholesterol is biphasic, and the rapid phase is complete within 10 min [15, 26].

Statistical methods. Differences between means were assessed by Student's *t*-test. One-way analysis of variance was used to compare three or more means. A P value of 0.05 or less was considered significant.

RESULTS

Plasma corticosterone and ACTH. Plasma corticosterone, the predominant steroid secreted by the rat adrenal cortex, was measured in TCDD-treated (50 μg/kg, single oral dose), pair-fed control (PFC), and *ad libitum*-fed control (ALC) rats. These rats, maintained on a fixed light/dark cycle under conditions that were carefully controlled to minimize stress, were killed near the beginning (AM) and end (PM) of the light phase (Fig. 1). Since pair feeding necessarily lagged 1 day behind the TCDD treatment, in one experiment ALC rats were maintained and killed in parallel to both TCDD and PFC groups (i.e. 1 day apart). Each group of rats was measured both under minimum stress conditions and 20 min after injection of a dose of ACTH which leads to maximum secretion of corticosterone [30]. Plasma corticosterone was extremely low in AM measurements of unstressed ALC rats, but was 8- to 10-fold higher in PM measurements (Table 1). This confirms previous reports of the diurnal rhythm of plasma corticosterone in unstressed ALC rats [14]. In AM measurements, TCDD treatment resulted in a 4-fold increase ($P < 0.05$) in the corticosterone level compared to ALC rats (Table 1). Comparable increases, however, were observed in PFC rats (Table 1, $P < 0.05$). In contrast, two sets of PM measurements using slightly different feeding schedules (Fig. 1) showed suppressions of plasma corticosterone by TCDD treatment of, respectively, 42% (Schedule A) or 29% (Schedule B) relative to ALC rats ($P < 0.05$). Levels of corticosterone in unstressed PFC rats that were killed 1 day later remained somewhat higher than levels in the corresponding group of ALC rats (Table 1).

Rats were stimulated by sufficient ACTH to test the effect of TCDD treatment on the maximum rate of adrenal steroid synthesis. Comparable increases in serum corticosterone were observed relative to the corresponding unstressed rats, irrespective of treatment (numbers in brackets, Table 1). Increases in the PM were smaller than in the AM, probably reflecting the higher unstressed corticosterone levels prior to ACTH administration. In one of the two experiments, the ACTH-induced increase in plasma

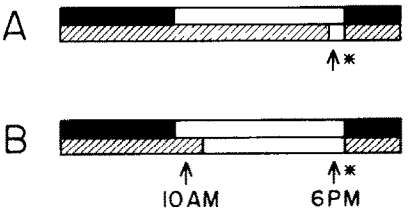

Fig. 1. Schematic diagrams of light/dark cycles and feeding schedules in relationship to when rats were killed. The white and black bars indicate the daily 12-hr light and 12-hr dark periods, respectively. The cross-hatched bars indicate when feed was available: Schedule A for 22 hr and Schedule B for 14 hr. The asterisk (*) represents the time of day when feed was placed in the cage; PFC rats normally consumed their entire daily ration of feed within a few hours after it was placed in the cage. Arrows indicate the time of day when rats were killed (10:00 AM and 6:00 PM).

Table 1. Effects of TCDD (50 μg/kg) on plasma corticosterone concentrations*

	Plasma corticosterone concentration (μg/dl)			
	ALC_T	TCDD-50	ALC_P	PFC
AM measurement				
Unstressed†	2.1 ± 0.7 (16)	7.9 ± 1.9‡ (15)	2.1 ± 1.1 (6)	5.3 ± 1.8‡ (6)
+ACTH†	26.2 ± 1.8 (6) [24.1]	37.6 ± 4.7‡ (6) [29.7]		30.9 ± 0.8‡ (6) [25.6]
PM measurement				
Unstressed†	23.9 ± 1.8 (10)	17.1 ± 1.4‡ (10)	15.7 ± 1.5 (10)	19.2 ± 1.9 (10)
+ACTH†	33.8 ± 1.6 (6) [12.8]	31.2 ± 2.9 (6) [13.4]		31.4 ± 0.8 (6) [14.6]
Unstressed§	23.6 ± 4.9 (3)	13.7 ± 4.3‡ (3)		28.1 ± 3.0 (3)
+ACTH§	43.6 ± 1.4 (3) [20.0]	29.5 ± 0.8‡ (3) [15.8]		54.8 ± 1.3‡ (3) [26.7]

* Rats were given 50 μg TCDD/kg or vehicle and killed 13 days later near the start (AM) or end (PM) of the daily light cycle. Rats were fed according to one of the schedules indicated in Fig. 1 and were either unstressed when killed or were given two units of ACTH 20 min before being killed. Pair-fed control (PFC) rats were killed 1 day after their TCDD-treated partners. ALC_T and ALC_P designate those *ad libitum* control rats killed simultaneously with the TCDD-treated and PFC rats respectively. Each value represents the mean ± standard error of the mean; the numbers in parentheses are the numbers of rats. Values in square brackets are the differences between the mean ACTH-stimulated plasma corticosterone concentration and the mean unstressed concentration.

† PM feeding on Schedule B in Fig. 1.

‡ TCDD or PFC significantly different from ALC ($P < 0.05$).

§ PM feeding on Schedule A in Fig. 1.

corticosterone concentration above the unstressed level was smaller in the TCDD-treated group than in the ALC group, but the differences were not significant. In each treatment group, corticosterone constituted greater than 85% of the total corticosteroids detectable in the plasma by HPLC, with or without ACTH treatment. TCDD treatment did not affect the proportion of the minor steroids 18-hydroxy-11-deoxycorticosterone and 11-deoxycorticosterone (data not shown).

In a separate experiment using a lower dose of TCDD (15 μg/kg) with feeding Schedule B, we observed pronounced decreases in PM corticosterone relative to both ALC (41%) and PFC (50%) values (Table 2). Further decreasing the dose of TCDD to 4.5 μg/kg results in a PM decrease of only 20% that was not significant [12]. Rats given these lower doses of TCDD (4.5 and 15 μg/kg) and their pair-fed controls failed to stimulate AM corticosterone levels relative to ALC rats (Ref. 12 and Table 2).

To determine whether TCDD treatment had caused a shift in the timing of peak secretion, plasma corticosterone was measured during an interval when periodicity is normally apparent (see Ref. 14 for a representative example). As shown in Table 3, TCDD treatment resulted in essentially constant corticosterone levels for an 8-hr period spanning the

Table 2. Effects of a low dose of TCDD on plasma corticosterone concentrations*

	Plasma corticosterone concentration (μg/dl)		
	ALC	TCDD-15	PFC
AM measurement			
Unstressed	2.7 ± 1.5 (9)	2.3 ± 0.6 (9)	4.1 ± 1.7 (8)
PM measurement			
Unstressed	18.7 ± 2.9 (9)	11.1 ± 1.6†‡ (9)	22.1 ± 1.8 (9)

* Rats were given 15 μg TCDD/kg or vehicle and killed 12 days later near the start (AM) or end (PM) of the daily light cycle (Fig. 1, Schedule B). Values are mean ± SEM (N).

† TCDD significantly different from ALC ($P < 0.05$).

‡ TCDD significantly different from PFC ($P < 0.05$).

Table 3. Diurnal cycle in plasma corticosterone concentrations 13 days after TCDD*

Time killed	Light/Dark schedule	Feed present	Plasma corticosterone concentration (μg/dl) TCDD-50	ALC
8:00 AM	Light	+	4.9 ± 1.5	2.1 ± 0.4
3:30 PM	Light	−	12.5 ± 3.3	
5:30 PM	Light	−	12.2 ± 2.4	17.7 ± 1.2
7:30 PM	Light	−	13.2 ± 2.6	
9:30 PM	Dark	+	13.0 ± 2.3	
11.30 PM	Dark	+	16.0 ± 4.5	

* Unstressed rats were killed 13 days after 50 μg TCDD/kg at the times shown. Lights were on from 8:00 AM to 8:00 PM. Feed was available from 8:00 PM to 10:00 AM (+) and was withdrawn at other times (−). Each value represents the mean ± SEM for five to six rats.

light/dark transition. These values were only about 3-fold higher than diurnal minimum (8:00 AM). In contrast, ALC rats showed a 9-fold increase in corticosterone between 8:00 AM and 5:30 PM. Thus, TCDD caused a partial suppression of the diurnal rhythm of serum corticosterone rather than a shift in the time of peak secretion.

The possibility that altered plasma ACTH levels in TCDD-treated rats caused the changes in plasma corticosterone was also examined. In control rats, there was a small increase from AM to PM measurements (Fig. 2). In other studies [14], higher PM ACTH levels have been observed, although the differences disappear with changes in diet [31]. The levels of plasma ACTH were not affected significantly by TCDD treatment and were changed only slightly by pair feeding.

Characterization of adrenal steroidogenic enzymes. Adrenal glands were removed from ALC, PFC, and TCDD-treated rats (50 μg/kg), 13 days after dosing.

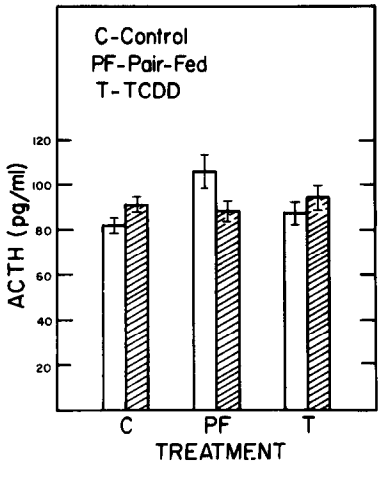

Fig. 2. Plasma ACTH levels in unstressed rats 13 days after TCDD. Plasma was obtained in the AM (open bars) and PM (cross-hatched bars) according to the lighting and feeding protocol in Fig. 1B. The dose of TCDD was 50 μg/kg. Each bar and associated vertical line represents the mean ± SEM (N = 15 for all values except PF, AM, N = 6).

Table 4 shows that no significant changes in total adrenal mass were observed for any of the three treatment groups. TCDD decreased both microsomal progesterone 21-hydroxylase activity and cytochrome P-450 content relative to values in microsomes from AFC and PFC rats. However, the absence of an effect of TCDD on the adrenal steroids quantitated in plasma indicates that this change is insufficient to cause accumulation of progesterone. Total mitochondrial cytochrome P-450 was unaffected by TCDD treatment, as were activities dependent on the individual cytochromes P-$450_{11\beta}$ and P-450_{scc} (11β-hydroxylation of 11-deoxycorticosterone and the conversion of 25-hydroxycholesterol to pregnenolone). Conversion of pregnenolone to progesterone was not measured since this activity is extremely rapid in adrenal cells and would require near complete inhibition to affect steroid production [15]. In short, no physiologically significant inhibition of corticosterone biosynthesis was detectable under these *in vitro* conditions.

Mitochondrial cholesterol. The cholesterol levels in isolated mitochondria reflect an *in vivo* steady state between the net rate for transfer of cholesterol to the mitochondria and the rate for subsequent metabolism [21]. Thus, increased cholesterol levels result from either a relative increase in uptake or a relative decrease in metabolism. Under unstressed conditions, free cholesterol levels in mitochondria from TCDD-treated rats showed small increases as compared to ALC values (24% in AM, 38% in PM) (Table 5). Increases of about two-thirds this size were seen in PFC rats (19% in AM, 26% in PM). Following ACTH treatment, adrenal mitochondrial cholesterol levels were increased only slightly in ALC and PFC rats (−4 to +13%) but were greatly increased following TCDD treatment (39 to 46%). In ACTH-administered TCDD-treated rats, adrenal mitochondrial cholesterol levels were 70–72% higher than in ACTH-treated ALC rats, regardless of the time of day.

The metabolism of endogenous adrenal mitochondrial cholesterol to pregnenolone consists of fast and slow phases differing by over 10-fold in their rates [15]. Since the fast phase of metabolism is complete within 5 min [26], about 90% of pregnenolone formed in a 10-min incubation period

Table 4. Effect of TCDD on adrenal weight and on cytochrome P-450 levels and steroidogenic enzyme activities in adrenal mitochondrial and microsomal fractions*

Rat treatment	Adrenal wt (mg wet wt)	Cytochrome P-450 (nmoles P-450/mg protein)		Enzyme activities (nmoles/mg protein/min)		Mitochondrial SCC of 25-hydroxycholesterol
		Mitochondria	Microsomes	Microsomal 21-hydroxylase	Mitochondrial 11β-hydroxylase	
ALC	26.8 ± 1.0	0.81 ± 0.03	0.61 ± 0.02	11.0 ± 0.6 (18)†	2.35 ± 0.1 (2.9)	1.2 ± 0.1
PFC	25.3 ± 0.7	0.83 ± 0.02	0.66 ± 0.04	13.5 ± 1 (20.5)	2.5 ± 0.25 (3.0)	
TCDD-50	23.6 ± 0.8	0.86 ± 0.05	0.42 ± 0.02‡	8.8 ± 0.4‡ (21)	2.75 ± 0.2 (3.2)	1.3 ± 0.1

* Control, pair-fed, and TCDD-treated rats (50 μg/kg) were killed 13 days post-treatment. Adrenals were defatted and weighed, and mitochondrial and microsomal fractions were prepared. Total cytochrome P-450 content was determined in each fraction by reduced-CO difference spectroscopy [24]. Mitochondrial 11β-hydroxylase and microsomal 21-hydroxylase activities were measured by the production of corticosterone from 11-deoxycorticosterone and 11-deoxycorticosterone from progesterone respectively. Side-chain cleavage of 25-hydroxycholesterol was determined by the production of pregnenolone. Each value represents the average ± SEM (N = 12 for adrenal weight and P-450 content, N = 4 for enzyme assays).

† Numbers in parentheses represent nmoles/nmole P-450.

‡ Significantly different from ALC and PFC ($P < 0.05$).

results from the reactive cholesterol pool of the isolated mitochondria. The amount of reactive cholesterol in mitochondria has been measured in the three treatment groups with both unstressed and ACTH-treated rats. Reactive mitochondrial cholesterol from unstressed ALC rats was 4-fold higher in the PM measurement than in the AM measurement, paralleling the diurnal change in plasma corticosterone levels (Fig. 3, basal). However, reactive cholesterol in mitochondria from unstressed, TCDD-treated rats was higher than that observed for ALC rats in both the AM (4.5-fold) and PM (1.3-fold) measurements. The reactive cholesterol pool after TCDD treatment differed little between the AM and the PM, but at both times this was substantially more than in mitochondria from PFC rats (2.2 and 1.7 times) (Fig. 3). Mitochondria from ACTH-stimulated rats exhibited substantially larger pools of reactive cholesterol in each group relative to mitochondria from the corresponding unstressed group (Fig. 3, +ACTH). However, in addition there was the nearly 2-fold increase in reactive cholesterol in mitochondria from TCDD-treated rats relative to both PFC and ALC rats.

In other studies, it has been shown that the reactive cholesterol pool is found in the inner mitochondrial membrane and that changes in the size of this pool are paralleled by changes in the association of cholesterol with cytochrome P-450_{scc} [16]. The spectral change induced by the binding of pregnenolone to cytochrome P-450_{scc} is proportional to the amount of cytochrome P-450 bound to cholesterol while the opposite spectral change induced by 20α,22*R*-dihydroxycholesterol is proportional to the level of cholesterol-free cytochrome P-450_{scc} [25]. Addition of the values obtained for cholesterol-bound and cholesterol-free cytochrome P-450_{scc} approximates the total levels of mitochondrial cytochrome P-450_{scc}. Table 6 shows that, in unstressed rats, the percentage of the total cytochrome P-450_{scc} that was complexed with cholesterol increased slightly in TCDD-treated rats (31%) as compared to both ALC rats (25%) and PFC rats (27%). Following ACTH treatment, complex formation increased to a much greater extent in TCDD-treated rats (45%) than in ALC (29%) or PFC rats (31%). As expected, these changes in complex formation parallel the changes in reactive cholesterol-derived cholesterol metabolism (Table 5).

DISCUSSION

TCDD produced substantial changes in plasma corticosterone without altering ACTH levels, probably through changing the responsiveness of the adrenal cortex to ACTH. The rate-limiting step in steroidogenesis is normally the rate of transport of cholesterol to cytochrome P-450_{scc} rather than the rate at which this enzyme converts cholesterol to pregnenolone [15, 17, 21]. The most notable effect of TCDD treatment on the adrenal cortex was an enhanced level of cholesterol in isolated mitochondria, particularly following ACTH treatment. This was associated with increased levels of a reactive pool of cholesterol and increased P-450_{scc}–cholesterol complex formation. These increases closely

Table 5. Effect of TCDD treatment on free cholesterol in isolated adrenal mitochondria*

Treatment	Mitochondrial free cholesterol (μg/mg protein)		
	Unstressed	+ACTH	% ACTH stimulation
AM			
Control	18.5 ± 0.7	19,5 ± 0.8	5
Pair-fed	22.0 ± 1.0†	23.0 ± 0.8†	5
TCDD	23.0 ± 1.0†	33.5 ± 0.8†‡§	46
PM			
Control	19.5 ± 0.7	22.0 ± 0.4	13
Pair-fed	24.5 ± 0.5†	23.5 ± 0.4	−4
TCDD	27.0 ± 1.7†	37.5 ± 2.5†‡§	39

* Mitochondria were prepared from rat adrenals obtained from ACTH-treated (+ACTH) or unstressed rats in the AM and PM according to the lighting and feeding protocol in Fig. 1B. Values represent the average ± SEM of four assays. Percent ACTH stimulation of mitochondrial free cholesterol was calculated from the ratio of +ACTH to unstressed within a given treatment group.

† TCDD or pair-fed significantly different from control ($P < 0.05$).

‡ TCDD significantly different from pair-fed ($P < 0.05$).

§ +ACTH significantly different from unstressed ($P < 0.05$).

resemble the effects upon adrenal mitochondria of *in vivo* treatment with an inhibitor of cholesterol SCC like aminoglutethimide. When cholesterol SCC is inhibited *in vivo* by aminoglutethimide, cholesterol entering adrenal mitochondria accumulates. This increase in mitochondrial cholesterol is maintained in the isolated mitochondria [17, 21]. ACTH increases the total and reactive pool of free cholesterol in adrenal mitochondria following aminoglutethimide treatment [17, 21] in much the same way that ACTH increased mitochondrial cholesterol following TCDD treatment.

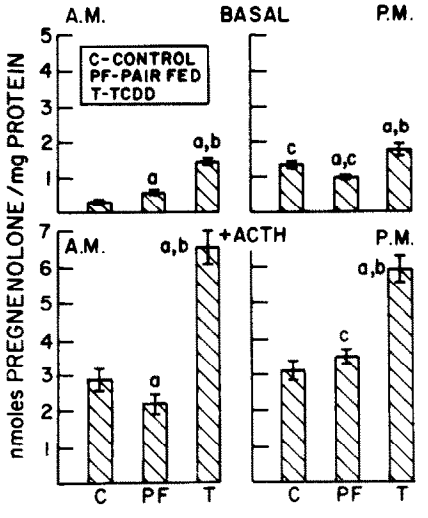

Fig. 3. Effect of TCDD on adrenal mitochondrial reactive cholesterol pools. Mitochondrial fractions were prepared from control, pair-fed, and TCDD-treated rats, 13 days post-treatment. Rats were killed in the morning (AM) or evening (PM) in accordance with a 12-hr light/dark schedule (Fig. 1, Schedule B), following an intraperitoneal injection of ACTH (two units/rat, 20 min) or no treatment (basal). Reactive cholesterol was measured by its conversion to pregnenolone within 10 min at 37°. Each bar and associated vertical line is the mean ± SEM of four assays. Key: (a) TCDD or pair-fed significantly different from control ($P < 0.05$); (b) TCDD significantly different from pair-fed ($P < 0.05$); and (c) PM value significantly different from AM value ($P < 0.05$).

The close similarity between the effects of TCDD treatment on adrenal mitochondrial cholesterol distribution and the *in vivo* action of aminoglutethimide suggests that TCDD may stimulate an inhibitor that is sufficiently water soluble or labile to be removed during mitochondrial isolation. Inhibition cannot be produced by TCDD itself, which does not directly inhibit cholesterol SCC (at 10^{-7} M) in isolated rat adrenal cells [32]. While direct evidence for a physiological inhibitor of cholesterol SCC activity in normal rats is limited, three types of cytosolic inhibitory activity upon mitochondrial cholesterol SCC have been reported, though none has been partially characterized [29. 33, 34]. Inhibition of cholesterol SCC may also result from a diminished supply of reducing equivalents to cytochrome $P\text{-}450_{scc}$ or restricted access to molecular oxygen.

TCDD-induced changes in adrenal sensitivity to ACTH cannot be the result of effects on adrenal weight, the amount of mitochondria in the adrenal cortex, mitochondrial cytochrome P-450 levels, or 11β-hydroxylase activity, which were all insensitive to TCDD. In addition, the decrease in microsomal 21-hydroxylase activity, associated with a TCDD-induced decline in microsomal cytochrome P-450, cannot account for the changes observed for plasma corticosterone, since TCDD treatment did not affect the distribution of plasma corticosteroids. The lack of any effect of TCDD treatment on the side-chain cleavage of added 25-hydroxycholesterol by isolated mitochondria or on total cytochrome $P\text{-}450_{scc}$ (assayed spectrally) indicates that TCDD did not produce a change in the concentration or activity of this enzyme that is retained in isolated mitochondria.

The actions of TCDD that result in, respectively, the AM stimulation and the PM decrease of plasma

Table 6. Effect of TCDD on cytochrome P-450$_{scc}$–cholesterol complex formation in adrenal mitochondria*

	Cytochrome P-450$_{scc}$ complex formation (nmoles/mg protein)			
	Cholesterol complex	Cholesterol free	Total	Percent complex
Control				
Basal	0.14	0.43	0.57	25
+ACTH	0.18	0.44	0.62	29
Pair-fed				
Basal	0.13	0.37	0.50	27
+ACTH	0.15	0.34	0.49	31
TCDD-treated				
Basal	0.16	0.37	0.53	31
+ACTH	0.25	0.31	0.56	45

* Adrenal cytochrome P-450$_{scc}$–cholesterol complex formation and free cytochrome were measured in whole mitochondria from unstressed (basal) and ACTH-treated (+ACTH) rats by the reverse-Type I and Type I spectral changes induced by pregnenolone and 20α,22R-dihydroxycholesterol respectively. Assuming an extinction coefficient for the difference spectra of 110 $mM^{-1}cm^{-1}$ [29], each value represents the average of two measurements that differed by less than 10%.

corticosterone can be clearly distinguished. TCDD treatment and the corresponding pair feeding were equally effective in producing the AM stimulation, indicating that this effect was due to nutritional deprivation. Pair feeding did not change the PM plasma corticosterone relative to levels in ALC rats, indicating that the TCDD suppression at this time was not due to diminished feed intake. In addition, a diminished dose of TCDD (15 μg/kg) abolished the AM stimulation but did not affect the PM. We attribute the PM suppression of corticosterone levels to TCDD-induced inhibition of cholesterol SCC. The ACTH-induced accumulation of mitochondrial cholesterol that is most clearly indicative of inhibition was not observed in PFC rats, indicating that this results directly from TCDD action. This inhibition of cholesterol SCC can only affect the rate of adrenal cholesterol metabolism when cholesterol transport is sufficiently stimulated so that it is no longer rate limiting. The greater steroid synthesis in the PM would fit this criterion more readily than the low AM synthesis since this rate increase probably results from enhanced cholesterol transport. Thus, although the TCDD-induced inhibition of cholesterol SCC was equally effective at both AM and PM measurements, TCDD suppression of steroidogenesis was only observed in PM measurements.

At this stage, far too little is known about the diurnal control of adrenal steroidogenesis to quantitatively relate cholesterol SCC activity to serum corticosterone levels. Several lines of evidence suggest that additional factors modulate adrenal responsiveness to ACTH during the diurnal cycle [35, 36], and these changes are at least in part retained in isolated cells [37]. Both ACTH and thyroxine appear necessary for the diurnal peak in corticosterone secretion, and this rhythmicity can be maintained when ACTH rhythmicity is prevented. Clearly TCDD may also affect the adrenal through these unknown regulatory processes. In addition, TCDD treatment substantially decreases plasma thyroxine concentrations [38, 39], and this decrease may contribute to the depression of peak corticosterone secretion.

It is likely that changes in adrenal physiology described in this paper result from a combination of direct effects of TCDD in adrenal cells and from changes in the adrenal secondary to effects of TCDD on other tissues [37]. TCDD-induced changes that may result from similar mechanisms also affect other steroid-secreting tissues. A severe androgenic deficiency occurs in male rats [12], and while there was no effect on estradiol in pregnant rats [40], substantial decreases in estradiol and pregnenolone have been reported in female monkeys [41]. Given the similarities in the control of cholesterol metabolism in steroidogenic organs [15], the effects of TCDD treatment similar to those in the adrenal may also occur in the testis, ovary, and placenta. Whether the decreases in sex hormone concentrations in TCDD-treated animals are caused by similar lesions remains to be determined.

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