

TCDD Alters Pituitary-Adrenal Function II: Evidence for Decreased Bioactivity of ACTH

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BESTERVELT, L. L., J. A. PITT, C. J. NOLAN AND W. N. PIPER. *TCDD alters pituitary-adrenal function II: Evidence for decreased bioactivity of ACTH.* NEUROTOXICOL TERATOL 15(6) 371-376, 1993.—The present study assessed the ability of primary cultures of rat anterior pituitary cells to secrete bioactive ACTH in the presence of 2, 3, 7, 8-tetrachlorodibenzo-*p*-dioxin (TCDD). The bioactivity of the secreted pituitary cell ACTH was determined by its ability to stimulate secretion of corticosterone from primary cultures of rat adrenal cells. ACTH from basal or CRH stimulated pituitary cells treated with TCDD was found to be less capable of stimulating corticosterone secretion from primary rat adrenal cell cultures than equimolar concentrations of ACTH purchased from a commercial supplier. Corticosterone secretion from adrenal cell cultures treated with ACTH from basal or CRH stimulated pituitary cell cultures exposed to TCDD was decreased by 60 and 70%, respectively. The decreased ability to stimulate corticosterone secretion can be overcome when extracts of ACTH from pituitary cell cultures treated with TCDD are supplemented with commercial ACTH. These findings indicate that TCDD may alter the bioactivity of secreted ACTH from the anterior pituitary gland.

2, 3, 7, 8-Tetrachlorodibenzo- <i>p</i> -dioxin	Adrenocorticotropin (ACTH)	Corticotropin releasing hormone (CRH)
Corticosterone	Pituitary gland	Adrenal gland

IN the preceding paper we demonstrated that 2, 3, 7, 8-tetrachlorodibenzo-*p*-dioxin (TCDD; 50 µg/kg; single, oral dose) causes an early (day 1) and persistent (day 14) increase in rat plasma ACTH concentrations. However, plasma corticosterone concentrations in the rat were not consistently increased or were actually decreased in spite of the increased plasma ACTH concentrations during this 14-day period. It has also been reported that TCDD-treatment decreases plasma corticosterone concentrations in the rat as early as day 4 after exposure (16). Under normal regulation, increased plasma ACTH concentrations should result in consistently elevated plasma corticosterone concentrations which was not observed, suggesting that TCDD may be able to decrease the capacity of the adrenal gland to respond to ACTH. In the preceding paper we also assessed the capacity of adrenal glands from rats treated with TCDD (50 µg/kg; single, oral dose) to respond to ACTH stimulation. It was discovered that adrenal responsiveness to exogenous ACTH was not changed by exposure to TCDD. Another possible mechanism for decreased plasma corticosterone concentrations in response to elevated plasma ACTH concentrations may be that TCDD directly effects the anterior pituitary to modify the synthesis and/or secretion of a less potent/bioactive form of ACTH. Secretion of a less

bioactive form of ACTH may explain the lack of a consistent increase in plasma corticosterone in response to elevated plasma ACTH in TCDD-treated rats. Because ACTH is the primary regulator of adrenal steroidogenesis, a shift from a more bioactive form to a less bioactive form would be expected to interfere with adrenal steroidogenesis. Therefore, it would be important to study the effects of TCDD on the bioactivity of ACTH. This study was designed to test the effects of TCDD on the ability of primary cultures of rat anterior pituitary cells to synthesize and secrete bioactive ACTH. The bioactivity of the secreted pituitary cell ACTH was assessed by its ability to stimulate secretion of corticosterone from primary cultures of rat adrenal cells.

METHOD

Primary Cultures of Anterior Pituitary Cells

After decapitation, pituitary glands were aseptically removed from 20–25 untreated Sprague-Dawley male rats (180–220 g) obtained from The Reproductive Sciences Program of The University of Michigan. Posterior pituitary tissue was separated from the anterior pituitary and discarded. Anterior pituitary tissue was quartered and enzymatically dispersed in

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20 ml Dulbecco's Modified Eagle's Medium (DMEM) (Gibco BRL; Grand Island, NY) using 0.35% type I collagenase (Sigma, St. Louis, MO) in a shaking water bath (37°C) for 60 min (21). The tissue fragments were allowed to settle for approximately 2 min and the media containing the collagenase was aspirated from the tissue fragments using a sterile flame-tipped Pasteur pipette. Complete DMEM (CDMEM: 2.2 g/l sodium bicarbonate, 2.5% bovine calf serum, 10% horse serum, 100 IU/ml penicillin, 100 mg/ml streptomycin, 0.1% bovine serum albumin, and 10 mM HEPES at pH 7.4) was added (5 ml) to the tissue fragments (15 ml). The resulting mixture was passed through a sterile flame-tipped Pasteur pipette to facilitate dissociation of the anterior pituitary cells from the pituitary gland's support membranes. The membranous debris was allowed to settle for approximately 2 min before media samples were removed, pooled, and placed into a sterile 50 ml conical tube. This process was repeated three times to recover a maximal number of anterior pituitary cells. The resulting pooled cell suspension was brought to a final volume of 45 ml with CDMEM, and centrifuged at $500 \times g$ for 20 min at 5°C to pellet the cells. The pellet was gently resuspended in 5 ml of CDMEM using a sterile flame-tipped Pasteur pipette. The cell suspension was brought to 20 ml with CDMEM and centrifuged as previously described. The cell concentration was determined using a hemocytometer, diluted to 1×10^5 cells/ml, and plated in 24 well 16 mm culture plates at 1 ml/well. The cells were maintained under standard culture conditions (95% air, 5% CO₂, 37°C) for the duration of the experiment. Before dosing, cells were allowed to attach for 4 days. TCDD was dissolved in dimethyl sulfoxide (DMSO) at a concentration of 10 mM just before addition to culture medium. Control samples were exposed to equal volumes of DMSO. Cell viability after TCDD treatment was assessed by trypan blue exclusion.

Primary Cultures of Adrenal Cells

After decapitation, adrenal glands were aseptically removed from 20–25 untreated Sprague-Dawley male rats (180–220 g) that were obtained from The Reproductive Sciences Program of The University of Michigan. Adrenal glands were trimmed of adhering fat and quartered. The quartered glands were enzymatically dispersed in 20 ml Dulbecco's Modified Eagle's Medium/Ham's F-12 (DMEM/F-12) (Gibco BRL; Grand Island, NY) containing 0.25% type I collagenase (Sigma; St. Louis, MO) in a shaking water bath for 30 min at 37°C (10,19,21). The cell suspension was allowed to settle for approximately 2 min before 20 ml of medium were removed and placed into a sterile 50 ml conical tube. Complete DMEM/F-12 (CDMEM/F-12: 2.2 g/l sodium bicarbonate, 2.5% bovine calf serum, 10% horse serum, 100 IU/ml penicillin, 100 mg/ml streptomycin, 0.1% bovine serum albumin, and 10 mM HEPES at pH 7.4) was added (5 ml) to approximately 10 ml of the original cell suspension. The resulting mixture was passed through a sterile flame-tipped Pasteur pipette to facilitate dissociation of the adrenal cells from the adrenal gland's support membranes. The membranous debris was allowed to settle for approximately 2 min before media samples were removed, pooled, and placed into a sterile 50 ml conical tube. This process was repeated three times to recover a maximal number of adrenal cells. The resulting pooled suspension was brought to a final volume of 45 ml with CDMEM/F-12, and centrifuged at $500 \times g$ for 20 min at 5°C to pellet the cells. The pellet was gently resuspended in CDMEM/F-12 and centrifuged as previously described. The

cell concentration was determined using a hemocytometer, diluted to 5×10^5 cells/ml, and plated in 35 mm petri dishes. Treatments were applied in serum-free media to prevent effects of sera from confounding the effects of TCDD. The ability of cells to exclude trypan blue was utilized following treatments in dishes to assess the effects of TCDD on cell viability.

ACTH Bioactivity Experiments

Primary cultures of rat anterior pituitary cells were initiated as described above. Cells were treated with TCDD (0, 10^{-9} , 10^{-11} , or 10^{-13} M) for 3 days. At the end of the treatment period, basal media samples from four petri dishes (1 ml from each petri dish) were pooled (total pooled media collected was 4 ml, and 200 μ l was removed for ACTH measurement). For the CRH (Peninsula; Belmont, CA) stimulated study, cells were treated with TCDD (0, 10^{-9} , 10^{-11} , or 10^{-13} M) for 3 days. After the treatment period, cells were rinsed with media (1 ml) and then stimulated with 10^{-7} M CRH for 5 h. This concentration of CRH has been shown to stimulate the synthesis and secretion of ACTH by cultured anterior pituitary cells (18,20,21). Following CRH stimulation, media samples from four petri dishes (1 ml from each petri dish) were pooled (total pooled media collected was 4 ml, and 200 μ l was removed for ACTH measurement).

After primary cultures of rat adrenal cells had reached confluency (7 days), they were rinsed with DMEM/F-12 (1 ml) and were challenged with basal pituitary media or CRH stimulated pituitary media (from controls and TCDD-treated pituitary cells) containing ACTH equivalent to 10^{-10} M by RIA for 5 h to determine the effect of TCDD on the bioactivity of secreted pituitary ACTH. Adrenal cells were also stimulated with ACTH (10^{-10} M; 1-39 human ACTH) purchased from Sigma Chemical Co., St. Louis, MO (exogenous ACTH) and compared with corticosterone secretion from basal and CRH stimulated pituitary ACTH media from TCDD-treated pituitary cells. Following stimulation, media samples were collected and stored at -20°C until measurement of corticosterone.

Several controls were used to ensure that media or hormonal treatments did not alter corticosterone secretion. The controls consisted of: a) equal volumes of adrenal medium (DMEM/F-12); b) equal volumes of pituitary medium, (DMEM); c) 10^{-10} M commercially purchased ACTH (the concentration of ACTH in pooled samples from TCDD-treated basal or TCDD-treated CRH stimulated pituitary media after RIA determination), and d) 10^{-10} M ACTH + 10^{-7} M CRH.

Adrenal cell cultures were also treated with pooled basal pituitary media from the 0, 10^{-9} , 10^{-11} , or 10^{-13} M TCDD-treated groups supplemented with 10^{-10} M ACTH purchased from Sigma Chemical Co., St. Louis, MO (exogenous ACTH) and stimulated for 5 h to ensure that TCDD did not interfere with ACTH stimulation of corticosterone secretion by adrenal cell cultures. Adrenal cell cultures were also stimulated with ACTH (10^{-10} M) purchased from Sigma Chemical Co., St. Louis, MO (exogenous ACTH), which served as a positive control. Following stimulation, media samples were collected and stored at -20°C until measurement of corticosterone. Cell viability was 90% or greater for all treatments tested.

ACTH and Corticosterone Measurements

ACTH was measured using a commercially available RIA kit (Diagnostic Products Corporation, Los Angeles, CA). The

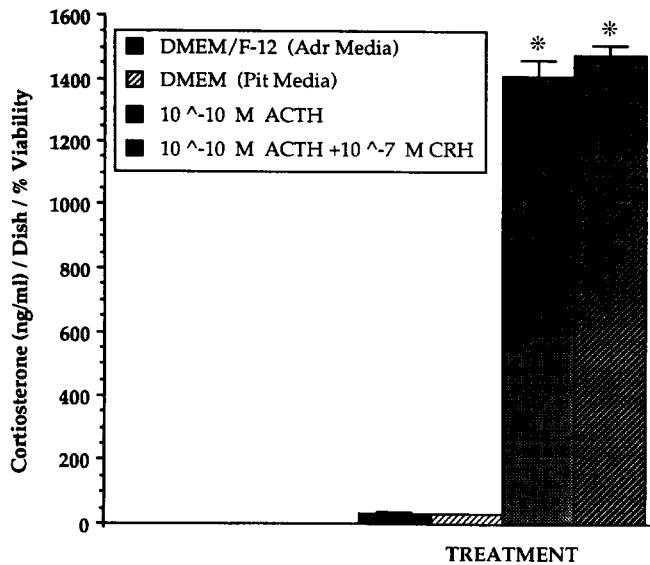


FIG. 1. Adrenal cell cultures were treated with DMEM/F-12 (adrenal control media), DMEM (pituitary media), 10^{-10} M ACTH or 10^{-10} M ACTH + 10^{-7} M CRH for 5 h to determine their effects on corticosterone secretion. Following stimulation, adrenal media samples were collected and corticosterone was measured by RIA. Values represent the mean \pm SEM for seven dishes. An asterisk denotes that the difference between media and hormonal treatments was statistically significant ($p < 0.05$).

ACTH antibody recognizes amino acids 1-24 from the N-terminus and crossreactivities ranged from 0.35% for α -MSH to nondetectable for Met- and Leu-enkephalin and β -endorphin. Intra- and interassay coefficients of variation for ACTH were 6% and 9%, respectively. Sensitivity of the ACTH RIA was 8 pg/ml. Corticosterone was also measured using a commercially available RIA kit (ICN Biochemicals Inc., Costa Mesa, CA). Sensitivity of the corticosterone RIA was 10 ng/ml and the intra- and interassay coefficients of variation were 7% and 8%, respectively. All assays were conducted as described by the manufacturer's protocol.

Statistical Analysis

Data were analyzed using one-way analysis of variance or Student's *t*-test to compare differences between means ($p < 0.05$).

RESULTS

Corticosterone secretion by primary rat adrenal cell cultures exposed to media, ACTH, or CRH is shown in Fig. 1. Basal corticosterone secretion is represented by DMEM/F-12 (adrenal medium) and served as a positive control. Pituitary medium (DMEM) did not change corticosterone secretion. Corticosterone concentrations were significantly increased in adrenal cell cultures that were treated with 10^{-10} M ACTH (exogenous ACTH) demonstrating that the adrenal cells respond to their appropriate hormonal stimulus. Because stimulated pituitary media samples contained both CRH and ACTH, it was important to determine corticosterone secretion by adrenal cells exposed to both of these hormones. Pituitary media samples containing 10^{-10} M ACTH and 10^{-7} M CRH did not differ in their ability to stimulate corticosterone secre-

tion from adrenal cell cultures treated with 10^{-10} M ACTH (Fig. 1), demonstrating that CRH had no effect on corticosterone secretion. Cell viability was 90% or greater for all treatments tested.

Corticosterone secretion by adrenal cells after treatment with pooled basal media samples from pituitary cultures treated with TCDD is shown in Fig. 2. In this experiment, basal media samples were pooled from TCDD-treated pituitary cells and ACTH concentrations were determined for each concentration of TCDD (10^{-9} - 10^{-13} M). The average concentration of ACTH for each of the pooled fractions was 10^{-10} M ACTH (five determinations for each pooled sample). Control basal media samples contained less than 10^{-14} M ACTH. Commercially purchased ACTH (10^{-10} M, exogenous ACTH) was used as the concentration to stimulate corticosterone secretion by adrenal cell cultures to serve as a positive control. Pooled pituitary media samples from all TCDD treatment groups (10^{-9} M, 10^{-11} M, and 10^{-13} M) stimulated secretion of 30-40% less corticosterone into the media by adrenal cell cultures than 10^{-10} M exogenous ACTH. Controls comprising basal pituitary media did not change corticosterone secretion. Cell viability was 90% or greater for all treatments tested.

Corticosterone secretion by adrenal cells after treatment with pooled pituitary media samples from CRH-stimulated TCDD-treatment groups is shown in Fig. 3. In this experiment, media samples were pooled from TCDD-treated pituitary cells that were stimulated with CRH and ACTH concentrations were determined for each concentration of TCDD (10^{-9} M, 10^{-11} M, and 10^{-13} M). The average concentration

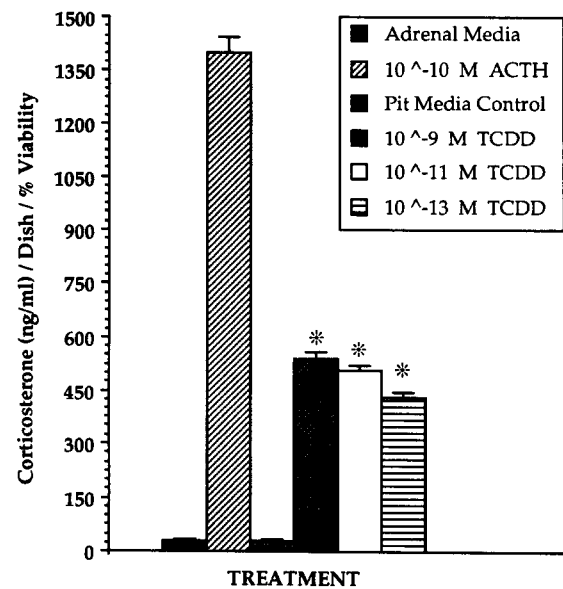


FIG. 2. Adrenal cell cultures were treated with pooled basal pituitary media samples from control ($< 10^{-14}$ M) or TCDD-treated pituitary cells (the ACTH concentration for the pooled media samples was 10^{-10} M) for 5 h to determine the effect of TCDD exposure on the bioactivity of secreted pituitary ACTH. Adrenal cells were also stimulated with commercially purchased 10^{-10} M (exogenous) ACTH and compared to corticosterone secretion from pooled basal TCDD-treated pituitary media. Following stimulation, media samples were collected and corticosterone was measured by RIA. Values represent the mean \pm SEM for seven dishes. An asterisk denotes that the difference between pooled basal TCDD-treated pituitary media and control values was statistically significant ($p < 0.05$).

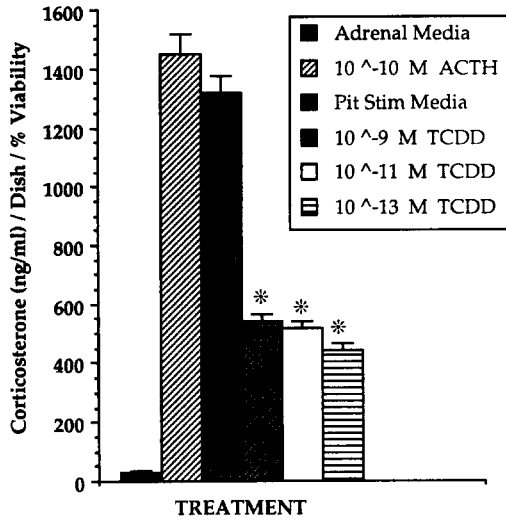


FIG. 3. Adrenal cell cultures were treated with pooled CRH stimulated pituitary media samples from control or treated pituitary cells (the ACTH concentration for the pooled media samples was 10^{-10} M) for 5 h to determine the effect of TCDD exposure on the bioactivity of secreted pituitary ACTH. Adrenal cells were also stimulated with commercially purchased 10^{-10} M (exogenous) ACTH and compared to corticosterone secretion from pooled TCDD-treated CRH stimulated pituitary media. Following stimulation, media samples were collected and corticosterone was measured by RIA. Values represent the mean \pm SEM for seven dishes. An asterisk denotes that the difference between pooled CRH stimulated TCDD-treated pituitary media and control values was statistically significant ($p < 0.05$).

of ACTH for each of the pooled fractions was 10^{-10} M ACTH (five determinations for each pooled sample). ACTH (10^{-10} M, exogenous ACTH) was used as a positive control. Pooled pituitary media samples, stimulated with CRH, from all TCDD-treatment groups (10^{-9} M, 10^{-11} M, and 10^{-13} M), caused 55%–70% less corticosterone to be secreted into the media by adrenal cell cultures than 10^{-10} M exogenous ACTH or controls consisting of pooled media samples from CRH stimulated pituitary media in the absence of TCDD. Note that pooled media samples from CRH stimulated pituitary cells stimulated corticosterone secretion by adrenal cell cultures as well as 10^{-10} M exogenous ACTH. Cell viability was 90% or greater for all treatments tested.

These results indicate that pooled basal or CRH stimulated pituitary media samples from TCDD-treated pituitary cell cultures are less capable of stimulating corticosterone secretion from adrenal cell cultures than equimolar, exogenous ACTH. To confirm that the decreased bioactivity of ACTH was not caused by the presence of TCDD in the pooled pituitary media samples, TCDD (10^{-9} M, 10^{-11} M, and 10^{-13} M)-treated pituitary media supplemented with 10^{-10} M exogenous ACTH was added to adrenal cell cultures and corticosterone secretion was assessed. The response of adrenal cell cultures to pituitary media samples supplemented with TCDD and 10^{-10} M exogenous ACTH is shown in Fig. 4. Corticosterone secretion caused by pituitary media samples supplemented with TCDD and 10^{-10} M exogenous ACTH did not differ from those supplemented with only 10^{-10} M exogenous ACTH, suggesting that TCDD contamination of pooled pituitary media samples was not responsible for the decreased bioactivity of ACTH in pituitary cell cultures that had been exposed to TCDD.

Furthermore, assay for TCDD in pooled media samples from pituitary cells treated with this toxicant by high resolution GC-low resolution MS (Dr. Matthew Zabic, Michigan State University; data not shown) yielded less than the level of detection of 3 ppt ($< 10^{-14}$ M), indicating that the presence of TCDD as a contaminant cannot explain the decreased bioactivity of ACTH.

DISCUSSION

The bioactivity of ACTH secreted from TCDD-treated basal or TCDD-treated CRH stimulated pituitary cell cultures was assessed in this study by testing the ability of each to stimulate secretion of corticosterone from primary rat adrenal cell cultures. We used the in vivo cell culture model to rule out the TCDD-induced weight loss as the cause of HPA perturbations observed in the previous paper (1a). Pooled basal pituitary media samples from TCDD-treated cells, measured for their ACTH concentrations, were found to be less capable of stimulating secretion of corticosterone by adrenal cells than equimolar, exogenous ACTH. Also, pooled pituitary media samples from TCDD-treated cells treated with CRH, measured for their ACTH concentrations, were also found to be less capable of stimulating secretion of corticosterone by adrenal cells than equimolar, exogenous ACTH. It was expected that pooled media samples from basal or CRH stimulated TCDD-treated pituitary cells would stimulate corticosterone secretion in amounts equivalent to equimolar, exogenously supplied 10^{-10} M ACTH. It was surprising to find that this did not happen. The inability to stimulate corticosterone secretion could be overcome when pituitary media samples from TCDD-treated cells were supplemented with additional exogenous ACTH. These findings support the hypothesis that TCDD may alter the bioactivity of ACTH secreted by the anterior pituitary gland and rule out TCDD-induced weight

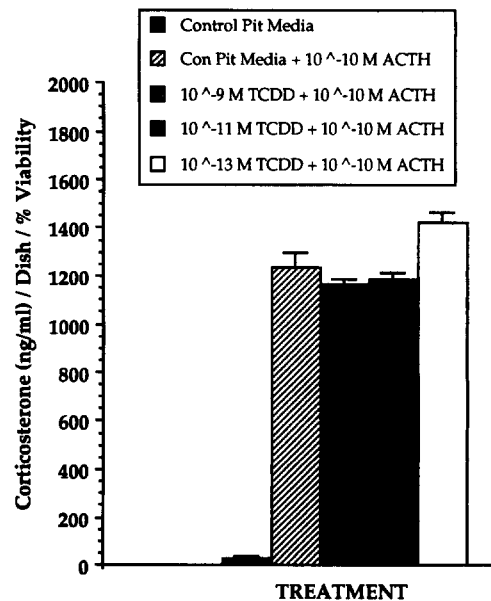


FIG. 4. Adrenal cell cultures were treated with pooled basal pituitary media supplemented with 10^{-10} M ACTH from control or TCDD-treated pituitary cells for 5 h. Following stimulation, media samples were collected and corticosterone was measured by RIA. Values represent the mean \pm SEM for seven dishes.

loss as a cause of the observed HPA alterations. It is important to remember that pooled media samples from control CRH stimulated pituitary cells stimulated corticosterone secretion by adrenal cell cultures as well as 10^{-10} M exogenous ACTH. The stimulation of corticosterone production by control pituitary secreted ACTH demonstrates that the harvesting procedure for pituitary cells did not cause an altered form of ACTH to be secreted.

ACTH is known to exist in several different processed and unprocessed forms with varying steroidogenic potencies. ACTH is synthesized from a large precursor molecule (31K pro-ACTH/endorphin). 31K Pro-ACTH/endorphin is post-translationally processed to yield different peptides. The 31K pro-ACTH/endorphin precursor is first proteolytically cleaved to a 22K ACTH biosynthetic intermediate, which is further cleaved to yield a 16K fragment and a 4.5K ACTH fragment (1,4,8,9,20). Each fragment of ACTH has been shown to have different steroidogenic capacities. The 4.5K ACTH is the most bioactive peptide of ACTH and has full steroidogenic potency as assessed by the synthesis and secretion of corticosterone by rat adrenal cell cultures (3-6,13,17,22,24). In contrast, the steroidogenic potencies of the 31K and 22K peptides have been found to be 100- and 300-fold less than the 4.5K fragment, respectively (2,6,7,11,17). The 31K and 22K ACTH peptides are both found in normal pituitaries, ACTH-secreting pituitary tumors, ectopic ACTH-secreting tumors, and in plasma and are believed to have little inherent biological activity (12,23,24). Therefore, it is possible that TCDD is able to preferentially influence the synthesis and secretion to the less processed forms of ACTH (31K and 22K) that have low adrenal steroidogenic potency.

Further support for this concept comes from studies conducted in rat anterior pituitary cells and mouse pituitary tumor cells. Secretion of these different ACTH peptide fragments varies under basal and CRH stimulated conditions (5,8,14). Under basal conditions, the less processed forms (31K and 22K) are predominant; whereas, stimulation with ACTH secretagogues such as CRH potentiates the secretion of the bioactive forms of ACTH (4.5K) (1,5,8,9,20). Furthermore, selective release of different ACTH peptide fragments has been demonstrated in rat anterior pituitary cell cultures (basal or CRH stimulated conditions) (8,9). It is believed that peptides secreted under basal conditions originate from different intracellular pools than peptides secreted under the influence of ACTH secretagogues. These different pools respond to different secretory signals to produce and secrete different end products (8,9). Thus, it is possible that during chronic TCDD treatment the newly synthesized peptides, which represent the unprocessed forms, are secreted in preference to the

more processed forms that would be less available for secretion. Therefore, this mechanism could explain the observation that pooled pituitary media samples from TCDD-treated cells were less capable of stimulating secretion of corticosterone from adrenal cells than RIA-measurable, equimolar concentrations of exogenous ACTH.

Another possibility is that TCDD is able to alter POMC processing into the various bioactive peptide fragments. TCDD could inhibit an early step in the processing of the POMC molecule promoting the accumulation of larger precursor-sized forms of ACTH (31K and 22K). Chronic administration of the drug haloperidol is believed to alter POMC processing with accumulation of larger, precursor-sized forms of ACTH (15). It is possible that POMC processing could be retarded during TCDD treatment, resulting in the accumulation of larger precursor-sized forms of ACTH. This process could involve the depletion of proteolytic enzymes involved in the biosynthetic pathway, because these enzymes appear to be secreted together with the ACTH peptides during exocytosis from the secretory vesicles (14). TCDD exposure may also directly inhibit the proteolytic enzymes involved in the biosynthetic pathway, resulting in the accumulation of larger precursor-sized forms of ACTH (31K and 22K).

In summary, media samples from basal or CRH stimulated pituitary cells exposed to TCDD, measured for their ACTH content, are less capable of stimulating corticosterone secretion from adrenal cell cultures than equimolar, commercially purchased (exogenous) ACTH. The decreased ability to stimulate corticosterone secretion from adrenal cells can be overcome when pituitary media samples treated with TCDD are supplemented with additional exogenous ACTH. These results suggest that TCDD may be able to alter the bioactivity of ACTH, perhaps by shifting the form of ACTH that is being processed from the anterior pituitary gland. Because ACTH is the primary regulator of adrenal steroidogenesis, a shift from the more bioactive form of ACTH to a less bioactive form would be expected to impair adrenal steroidogenesis.

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