Comparison of accumulation and altered steroid secretion by placental tissue treated with TCDD and natural mixture of PCDDs–PCDFs

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Explants of human placental tissue harvested immediately after expulsion were used to determine differences between accumulation of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and polychlorinated dibenzo-*p*-dioxin (PCDD)–polychlorinated dibenzo-*p*-furans (PCDF) environmental mixture, and their influence on placental steroidogenesis. Explants were cultured *in vitro* for 5 days in media supplemented each day with either TCDD or a mixture of PCDD–PCDF. Media were collected every day for steroid content analysis by radioimmunoassay. At 24 h after the last treatment, the tissue was frozen for further analysis of the content of TCDD or other congeners present in the mixture. Determinations of TCDD and all 17 PCDDs and PCDFs were performed using gas chromatography equipped with DB-5 MS and DB-17 capillary columns. In the control tissue, the amounts of both TCDD and mixture components were close to the limit of detection of the method. In the treated tissue, the TCDD accumulation was 94% of the total exposure to TCDD. The most toxic congeners 2,3,7,8-TCDD, 2,3,7,8-tetrachlorodibenzofuran, 1,2,3,7,8-pentachlorodibenzo-*p*-dioxin (PeCDD), 1,2,3,7,8-pentachloro-

dibenzo-*p*-furans (PeCDF) and 2,3,4,7,8-PeCDF showed the highest accumulation, which covered > 50% of the total toxic equivalents present in this mixture. During the first 3 days of exposure to TCDD there was no effect on the conversion of dehydroepiandrosterone to oestradiol, whereas on days 4 and 5 of exposure, a twofold decrease in oestradiol secretion was observed. However, a small but significant increase in oestradiol secretion was noted at all times of exposure to the PCDD–PCDF mixture. All observed changes in oestradiol secretion were not accompanied by changes in progesterone secretion after exposure to TCDD or the PCDD–PCDF mixture. In conclusion, a high accumulation of TCDD in the placental tissue resulted in a decrease in oestradiol secretion and *in vivo* this could result in a decrease in blood flow through the placenta. From the mixture, PeCDD and PeCDF in the higher amount accumulated in the placental tissue caused an increase in oestrogen secretion and as a consequence could activate oxytocin secretion from the pituitary and early pregnancy outcome.

Introduction

2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and related compounds elicit a diverse spectrum of toxic responses. As these compounds are lipophilic and difficult to metabolize, any environmental exposure of living organisms to these congeners results in their accumulation in the fat tissue and bioconcentration in humans via the food chain. In addition, these compounds are able to pass through the human placenta (Koppe *et al.*, 1992). Taking into account fat solubility of these compounds

and the maternal origin of 10–20% of fetal fatty acids, polychlorinated biphenyls (PCBs) and hexachlorobenzene (HCB) may impair the development of the fetus (Manchester and Jacobsy, 1984). Exposure of humans to halogenated aromatic hydrocarbons, such as polychlorinated biphenyls, is associated with adverse pregnancy outcomes, including intrauterine growth retardation, congenital structural anomalies and cognitive developmental deficits. Chen *et al.* (1992) and Rogan *et al.* (1988) observed impaired development of the fetus in women exposed to PCB, dioxins and dibenzofurans. Therefore, these compounds represent a serious health risk, especially to the fetus and infants, whose enzymatic and metabolic systems are not yet mature.

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There have been few studies on the direct effects of dioxin on placental steroidogenesis. A study by Augustowska *et al.* (2003) involving the primary culture of cells isolated from placental cotyledons harvested immediately after expulsion showed a difference between the action of pure TCDD and dioxin mixture on placental steroid secretion. This difference was possibly due to an additional effect of pentachlorodibenzo-*p*-dioxin (PeCDD) and pentachlorodibenzo-*p*-furan (PeCDF), which accounted for > 50% of the total toxic equivalents (TEQ) present in this mixture. However, it is not clear whether particular congeners present in the mixture accumulate in the same amounts. The present study used *ex vivo* culture of pieces of placental tissue in an attempt to understand the differences in the action of TCDD and dioxin mixture on placental steroidogenesis. The following were considered: (i) accumulation of pure TCDD and a mixture of polychlorinated dibenzo-*p*-dioxin (PCDD)–polychlorinated dibenzo-*p*-furans (PCDF) in the placental tissue; and (ii) the effects of repeated exposure to these compounds on placental steroidogenesis. This cultured tissue is more physiological than cell dispersed or cell lines because of the intact structure.

Materials and Methods

Reagents

Medium M199, PBS, penicillin, trypsin and calf serum were purchased from the Laboratory of Vaccines (Lublin). Dehydroepiandrosterone, cholesterol and antibiotic antimetabolic solution ($\times 100$) were obtained from Sigma Chemical Co. (St Louis, MO). 2,3,7,8-TCDD solutions were prepared by the dilution of evaporated, concentrated toluene standard (Promochem, Wesel) in dimethyl sulphoxide (DMSO). PCDD and PCDF natural congener mixture in DMSO was prepared by toluene Soxhlet extraction of 10 g fly ash sample collected from the hospital waste incinerator and Alumina column cleaned-up according to Grochowalski (1998). This mixture contained TCDD, PeCDD, hexachlorodibenzo-*p*-dioxin (1,2,3,4,7,8-HxCDD, 1,2,3,6,7,8-HxCDD and 1,2,3,7,8,9-HxCDD), 1,2,3,4,6,7,8-heptachlorodibenzo-*p*-dioxin (HpCDD), octachlorodibenzo-*p*-dioxin (OCDD), 2,3,7,8-tetrachlorodibenzofuran (TCDF), pentachlorodibenzofuran (1,2,3,7,8-PeCDF and 2,3,4,7,8-PeCDF), hexachlorodibenzofuran (1,2,3,4,7,8-HxCDF, 1,2,3,6,7,8-HxCDF, 2,3,4,6,7,8-HxCDF and 1,2,3,7,8,9-HxCDF), heptachlorodibenzofuran (1,2,3,4,6,7,8-HpCDF and 1,2,3,4,7,8,9-HpCDF) and octachlorodibenzofuran (OCDF). The concentration of all 17 toxic congeners was reported and TEQ was calculated as 27.7 $\mu\text{g TEQ kg}^{-1}$ of fly ash. After extract clean-up using standard procedure on Alumina, the solvent was exchanged with DMSO to obtain the stock solution at a concentration of 10 ng TEQ ml^{-1} . Working solutions were prepared by a dilution of the stock solution with DMSO to obtain

appropriate PCDD–PCDF concentrations just before addition to the culture medium.

Semi-permeable membranes for dialysis (2.5 cm $\times$ 20.0 cm polyethylene foil of 80.0 μm wall thickness) purchased from Exposmeter AB Trehörningen 34SE-929 26 Tavelsjö were used after rinsing with hexane.

Culture

Placental tissue was collected in a gynaecological hospital in Krakow, Poland where the clinical information on pregnancy outcomes was obtained. Collection of placentae and recording of clinical histories followed previously established protocols that had ethical approval by a local institutional review board. Patients gave their informed consent to the study. Clinical information recorded on each pregnancy included: smoking history, neonatal mortality and pregnancy outcome. Normal term (weeks 40–42 of gestation) placentae from non-smoking women were collected for the experiment. Immediately after expulsion of the placenta, placental cotyledons were harvested, placed in ice-cold PBS and transported to the laboratory. The tissue was cut into 10–15 mg pieces and incubated in Erlenmeyer flasks containing 3 ml M199 medium according to Gregoraszczyk (1990). The flasks were incubated at 37°C with constant shaking at 70 r.p.m. for 5 days for better penetration of the media, oxygen and also chemicals. The method of culture used in the present study permits for the examination of chemical accumulation in the tissue. This method was used to measure TCDD (Grochowalski *et al.*, 2000a) and PCB (Gregoraszczyk *et al.*, 2003) accumulation in the follicular wall. The percentage of proliferating cells as measured using the Molecular Immunology Borstel (MIB-I) labelling index showed 81.2% of proliferating cells in 6 days of culture, indicating that the model is suitable. Because of the intact structure present *in vitro*, there is a benefit to culture of pieces of placenta. This type of *ex vivo* culture has been successfully used for ovarian and breast cancer tissue (Devine *et al.*, 2002; Fruehauf, 2002). Ovarian tissues have been successfully incubated for up to 50 days without visible signs of necrosis (Blandau *et al.*, 1965).

Experimental procedure

One placental cotyledon (approximately 50 g) was removed from the underlying fibrous elements. Soft villous material from the maternal surface was cut away from connective tissue and vessels, according to the procedure described by Kliman *et al.* (1986). In the control culture, placental tissue was cultured in Parker medium (M199) supplemented with 5% calf serum, whereas in the experimental groups, TCDD or PCDD–PCDF mixture was added daily for 4 days at a dosage of 40 pg TEQ ml^{-1} from day 0 to day 4 of culture. The media in the control and experimental groups were changed

every day, and collected and frozen for steroid analysis. The total dose was 600 pg TEQ. At 24 h after the last treatment (day 5), tissue was frozen for further analysis of accumulation of TCDD and mixture components in the tissue, and the culture medium was frozen for analysis of steroid concentration. Experiments were repeated five times using cotyledons collected from five different patients.

Dehydroepiandrosterone (DHEA; 1 ng ml⁻¹), a natural substrate for oestradiol synthesis in the placental tissue was used to measure the effect of dioxin on oestradiol production, and pregnenolone (10 µg ml⁻¹) was used for its influence on progesterone secretion.

Chemical analysis

Sample clean-up was performed using semi-permeable membrane, carbon column, acid-base silica and Alumina column separation according to Grochowalski *et al.* (2002). Determination of TCDD and all 17 PCDDs and PCDFs was performed using gas chromatography equipped with DB-5 MS and DB-17 capillary columns (60.0 m in length, 0.25 mm inside diameter and 0.25 µm of stationary phase film). For PCDD and PCDF congener detection, double fragmentation mass spectrometric detector based on Finnigan MAT GCQplus GC-MS/MS instruments operated in Collisionally Induced Dissociation was used. Identification and confirmation was carried out on the principle of characteristic mass spectral data obtained from electron primary ions (electron impact ionization) and helium atom collision secondary ions (MS/MS mode). The limit of detection for PCDDs and PCDFs was 0.01 pg g⁻¹ for 2,3,7,8-TCDD and 2,3,7,8-TCDF (0.01 ppt) and 0.1 pg kg⁻¹ for OCDD and OCDF (0.1 ppt), respectively, in various sample matrices. In analytical work, 16 ¹³C]-PCDD-PCDFs were used as internal standards and for clean-up and recovery measurement.

TEQ calculation

Calculation of TEQ of the sample is based on the 2,3,7,8-TCDD toxicity equivalent factors (TEF). The toxic or dioxin equivalent (TEQ) for a mixture was defined as the sum of the concentration of individual PCDD-PCDF times their corresponding individual toxic equivalency factors (TEF_i) which were initially derived from enzymatic induction potency ratios (EC₅₀ [TCDD]/EC₅₀ [test compound]) using TCDD the reference toxin (that is TEF = 1) according to Safe (1996).

Steroid concentration analysis

Progesterone, testosterone and oestradiol were determined by radioimmunoassay using Spectra kits (Orion, Diagnostics) supplied by Polatom (Swierk). The limit of sensitivity for the progesterone assay was 94 pg ml⁻¹. The interassay and intra-assay coefficients of variation were 5.8

and 2.9%, respectively. The mean recoveries were 95.1–103.7%. The crossreactivity with pregnenolone was 2.9%. All other steroids tested (5β-dihydroprogesterone, 20β-hydroxyprogesterone, corticosterone, testosterone and oestrone) showed <1% crossreactivity. The limit of sensitivity for testosterone was 5 pg ml⁻¹ and the interassay and intra-assay coefficients of variation were 5.4 and 5.3%, respectively. The mean recoveries were 84.2–121.7%. Crossreactivity with 5α-dihydrotestosterone was 4.5%. All other steroids tested (methyltestosterone, androstendione, progesterone and oestradiol) showed <0.5% crossreactivity. The limit of sensitivity for oestrogen assay was 5 pg and the interassay and intra-assay coefficients of variation were 10.28 and 2.9%, respectively. The mean recoveries were 85.6–108.9%. The crossreactivity with ethinyl oestradiol was 1.4%. All other steroids tested (oestrone, oestriol, progesterone, testosterone and corticosterone) showed <1% crossreactivity.

Statistical analysis

All data points are expressed as means ± SEM. There were three replicates per treatment. Experiments were repeated five times using cotyledons collected from five different patients. Since the variation between the experiments was small, these results were analysed by ANOVA followed by Duncan's new multiple range test.

Results

Accumulation of TCDD and PCDD-PCDF mixture

In the control culture, the concentrations of both TCDD and mixture components were close to the limit of detection. In the experimental culture, the amount of TCDD accumulation was 94% of the total exposure.

The accumulation of particular congeners of the mixture are shown (Table 1), expressed as pg g⁻¹ tissue and as a percentage of accumulation of the total dose. The most toxic congeners, 2,3,7,8-TCDD (41.6%), 2,3,7,8-TCDF (26.31%), 1,2,3,7,8-PeCDD (13.05%), 1,2,3,7,8-PeCDF (9.18%) and 2,3,4,7,8-PeCDF (16.63%), showed the highest accumulation, which accounted for > 50% of the TEQ present in this mixture. The accumulation of the remaining congeners was about 10% of the total dose.

Effect of repeated doses of TCDD on oestradiol and progesterone secretion

A significant increase in oestradiol secretion was noted on day 2 of exposure to 40 pg TCDD ml⁻¹ ($P < 0.01$). The situation changed with increased exposure to TCDD: a twofold decrease in oestradiol secretion was observed on days 3 and 5 ($P < 0.01$; Fig. 1). TCDD had no effect on the conversion of pregnenolone to progesterone (Fig. 2).

Table 1. Concentration of individual PCDD–PCDF in placental tissue exposed *ex vivo* to PCDD–PCDF mixture administered each day for 4 days at a dose of 40 pg TEQ ml⁻¹

Lp I	Congeners	m _i (ng ml ⁻¹)	Accumulation (ng g ⁻¹)	m _i × TEF _i (ng g ⁻¹)	Percentage of accumulation
1	2,3,7,8 TCDD	0.28	0.101	0.101	36.07
2	1,2,3,7,8 PeCDD	1.74	0.313	0.313	17.98
3	1,2,3,4,7,8 HxCDD	2.93	0.312	0.031	10.64
4	1,2,3,6,7,8 HxCDD	4.53	0.49	0.049	10.81
5	1,2,3,7,8, HxCDD	5.03	0.479	0.048	9.52
6	1,2,3,4,6,7,8 HpCDD	27.58	2.15	0.021	7.79
7	OCDD	27.18	2.779	0.000	10.22
8	2,3,7,8 TCDF	1.14	0.283	0.028	24.82
9	1,2,3,7,8 PeCDF	3.54	0.469	0.023	13.24
10	2,3,4,7,8 PeCDF	6.69	1.52	0.760	22.72
11	1,2,3,4,7,8 HxCDF	9.2	1.238	0.124	13.45
12	1,2,3,6,7,8 HxCDF	8.98	0.659	0.066	7.33
13	2,3,4,6,7,8 HxCDF	13.64	1.914	0.191	14.03
14	1,2,3,7,8,9 HxCDF	0.83	0.104	0.010	12.38
15	1,2,3,4,6,7,8 HpCDF	33.41	2.123	0.021	6.35
16	1,2,3,4,7,8,9 HpCDF	5.7	0.557	0.006	9.77
17	OCDF	11.92	0.941	0.000	7.9

m_i: mass of individual congeners; PCDD–PCDF: polychlorinated benzo-*p*-dioxin–polychlorinated dibenzo-*p*-furan; TEF_i: toxic equivalency factors; TEQ: total toxic equivalents.

Fig. 1. Effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD; 40 pg ml⁻¹) and polychlorinated dibenzo-*p*-dioxin–polychlorinated dibenzo-*p*-furan (PCDD–PCDF) mixture (40 pg ml⁻¹) on conversion of dehydroepiandrosterone (DHEA; 1 ng ml⁻¹) to oestradiol. All values are the mean ± SE and expressed as percentage secretion, with control as 100%. ***P* < 0.01 compared with the control. ■: DHEA; ▨: DHEA + PCDD–PCDF; □: DHEA + TCDD. **Fig. 2.** Effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD; 40 pg ml⁻¹) and polychlorinated dibenzo-*p*-dioxin–polychlorinated dibenzo-*p*-furan (PCDD–PCDF) mixture (40 pg ml⁻¹) on conversion of pregnenolone (10 µg ml⁻¹) to progesterone. All values are the mean ± SE and expressed as percentage secretion, with control as 100%. ■: pregnenolone; ▨: pregnenolone + PCDD–PCDF; □: pregnenolone + TCDD.

Effect of repeated doses of PCDD–PCDF on oestradiol and progesterone secretion

A small, but significant increase in oestradiol secretion was noted on all days of exposure to 40 pg TEQ ml⁻¹ PCDD–PCDF mixture (Fig. 1). After exposure to the mixture all changes observed in oestradiol secretion were not accompanied by changes in progesterone secretion (Fig. 2).

Discussion

Chronic exposure to persistent endocrine disruptors results in their bioaccumulation in increasing concentrations in living organisms. The present study clearly showed differences between the accumulation of pure TCDD and a dioxin mixture. From the congeners present in the mixture, the most toxic compounds, 2,3,7,8-TCDD and 2,3,7,8-TCDF, showed the highest accumulation.

Higher chlorinated dioxins and furans are more lipophilic than lower chlorinated compounds such as TCDD. Compounds that are more lipophilic may result in lower accumulation of TCDD in the mixture of all 17 PCDDs and PCDFs than that of pure TCDD added to the samples. The sample that was treated with the PCDD–PCDF mixture showed a distribution of all 17 congeners typical of that found in the human placental tissue. This finding indicates that during the experiment all of the 17 individual congeners present in the mixture accumulate with respect to their individual solubility in fat. Moreover, some congeners may be metabolized more effectively than TCDD or TCDF. Grochowalski and Chrzęszcz (2000) reported that fat tissue of fish contains mostly 2,3,7,8-substituted PCDDs and PCDFs which are less effectively metabolized. The explanation of the effect of different accumulation values for the 17 PCDDs and PCDFs may require further study with higher as well as lower concentrations of PCDD and PCDF in the mixture.

There is evidence that these toxic compounds accumulate in the human placenta (Lucier *et al.*, 1990; Schecter *et al.*, 1996, 1998; Grochowalski *et al.*, 2000b). Koppe *et al.* (1992) showed that the placenta transports dioxins and furans from mother to fetus, and this is probably related to the fatty acid transport. Between 10 and 20% of fatty acids in a fetus at term are of maternal origin. In adipose tissue of children that died in the early neonatal period, concentrations of 25% were found of three dioxin and furan congeners 1,2,3,7,8-PeCDD, 1,2,3,6,7,8-HpCDD and 2,3,4,7,8-PeCDF in relation to the mean concentration of these congeners in the fat of 14 samples of breast milk.

Hagenmaier *et al.* (1990) noted the highest deposition for 2,3,7,8-TCDD and 1,2,3,7,8-PCDD in the infant marmoset monkey (*Callithrix jacchus*) after a lactation period of 33 days the mother of which has been subcutaneously administered a defined mixture of PCDD and PCDF 11 weeks before the delivery. This finding is in accordance with the results of the present study showing the highest accumulation of 2,3,7,8-TCDD and 2,3,7,8-TCDF in the placental tissue exposed *in vitro* to the mixture of dioxins. Our previous data indicated a link between the increased concentration of the most toxic PCBs in the placenta and cigarette smoking during pregnancy and a tendency for an increase in PCB 156, PCB 114 and PCB124 in placentae from abnormal pregnancy outcomes. DeKoning and Karmaus (2000) showed that on a lipid basis, the highest concentration of PCB in placenta was 2.8-fold higher than the highest concentration of PCB in breast milk and suggest that PCBs may be capable of crossing the placenta to a greater extent than previously believed.

As a consequence of the accumulation, an anti-oestrogenic action of TCDD was noted starting from day 3 of exposure and oestrogenic action of the mixture at all times of exposition. The human syncytial trophoblast is known to serve several roles in pregnancy. The human

placenta expresses high aromatase activity. An alteration in aromatase function in the uterus has been shown to permanently affect human embryos (Simpson *et al.*, 1994). TCDD is well known as an anti-oestrogen that enhances oestrogen metabolism (Spink *et al.*, 1990) and decreases oestrogen-induced responses such as increased uterine mass (Pohjanvirta and Tuomisto, 1994), cell proliferation (Sun and Safe, 1997) and the regulation of receptors related to the epidermal growth factor (Astroff *et al.*, 1990), oestrogen (Wang *et al.*, 1993) and progesterone (Harper *et al.*, 1994).

However, humans are exposed daily to mixtures of chemicals, rather than to individual chemicals. From a public health perspective, it is most relevant to answer the question of whether the components in a mixture interact in a way that results in an increase in their overall effect compared with the sum of the effects of the individual components. Surprisingly, only a few studies have examined the effects of PCDF on reproduction and development. The results of the present study are the first to show the effects of repeated exposure to a mixture PCDD–PCDF that occurs in the environment on placental steroidogenesis. In contrast to the action of TCDD, the mixture of dioxin–furans showed small but significant oestrogenic action. It was noted that there was 50% more oestradiol in the culture medium under the influence of this mixture. In fact, oestrogen regulates low-density lipoprotein (LDL) uptake and P450_{scc} and, thus, apparently is involved in generating the substrate for progesterone production within the placenta and there are reports of an increase in the oestrogen:progesterone ratio in the amniotic fluid of women during labour (Romero *et al.*, 1988). Placental microsomes and mitochondria incubated with a single dose of oestrogen showed a decrease in progesterone formation by inhibition of 3- β HSD (Depp *et al.*, 1973; Ferre *et al.*, 1975; Yoshida *et al.*, 1989). Genti-Raimondi *et al.* (1983) showed that physiological doses of oestrogen had a stimulatory effect on the conversion of pregnenolone to progesterone; however, supraphysiological doses had an inhibitory effect.

The lack of action of the mixture on progesterone secretion and the oestrogenic action of the mixture on placental tissue observed in the present study should be taken into consideration in view of the study by Chen *et al.* (2001), who examined the transfer of polychlorinated dibenzo-*p*-dioxins, dibenzofurans (PCDFs) and non-ortho biphenyls to offspring and placenta. They showed a dose-dependent increase in hepatic sequestration with TCDD, PeCDD, 4-PeCDF and OCDF. TCDD and three PCBs reached equilibration between the fetus and placenta.

The results of the present study are in accordance with those of McMurry and Dickerson (2001), who showed that the mixture of six different endocrine disrupters induced effects that were very different from either or both mixture components, indicating the lack of

predictability of chemicals when combined in a mixture. In addition, Chu *et al.* (2001) indicated that the mixture of PCBs and TCDD may be additive or antagonistic depending on the dose and endpoints measured. The effects of a mixture of dioxins may be additive or antagonistic depending on the dose. For the purpose of predicting mixture effects, knowledge of the mechanism of action and toxicokinetics is required. Because oestriol may affect uterine cystyl aminopeptidase gene expression (Darne *et al.*, 1987), it could contribute to the progressive increase in uterine responsiveness to xenobiotics in primate pregnancy during the third trimester of gestation, and its measurements may be of predictive value in delineating patients at risk of premature delivery (Darne *et al.*, 1987; Romero *et al.*, 1988).

Information concerning mechanisms of dioxin mixture action on placental cells is scarce. These preliminary experiments reported here indicate that further studies on the induction of CYP isozymes mRNA in the human placenta by these compounds are needed to understand the differences in the action of TCDD and dioxin mixture on placental steroidogenesis.

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