Inhibition of basal and stimulated progesterone synthesis by dichlorodiphenyldichloroethylene and methoxychlor in a stable pig granulosa cell line

N. K. Crellin¹, H. G. Kang², C. L. Swan³ and P. J. Chedrese^{3*}

¹Toxicology Centre, University of Saskatchewan, Saskatoon SK, Canada S7N 5B3;

²National Veterinary Research and Quarantine Service, Kyung-Gi province, Republic of Korea; and ³Reproductive Biology Research Unit, Department of Obstetrics, Gynecology and Reproductive Sciences, University of Saskatchewan, Saskatoon SK, Canada S7N 0W8

The effects of the insecticide dichlorodiphenyldichloroethylene (DDE) and methoxychlor in a stable pig granulosa cell line, JC-410, were investigated. The studies of DDE and methoxychlor were conducted in combination with studies of cholera toxin, the protein kinase A activator that stimulates cAMP and progesterone synthesis and gene expression of P450 cholesterol side chain cleavage (P450scc), which converts cholesterol to pregnenolone. Administration of DDE at 3000 and 10 000 ng ml⁻¹ was found to decrease progesterone synthesis 0.49- and 0.25-fold, respectively, and to block the stimulatory effect of 100 ng cholera toxin ml⁻¹, after 24 h incubation. At 1–100 ng ml⁻¹, methoxychlor did not affect progesterone synthesis after 48 h incubation. However, 1000 ng methoxychlor ml⁻¹ decreased progesterone synthesis 0.32-fold, and both 100 and 1000 ng methoxychlor ml⁻¹ blocked the stimulatory effect of cholera toxin. At 3000 and 10 000 ng ml⁻¹, DDE decreased cAMP synthesis 0.66- and 0.36-fold, respectively. At 300, 3000 and 10 000 ng

ml⁻¹, DDE also decreased cholera toxin-stimulated cAMP synthesis 0.84-, 0.68-, and 0.52-fold, respectively. Administration of 1–100 ng methoxychlor ml⁻¹ did not affect basal or cholera toxin-stimulated cAMP synthesis. Cholera toxin increased P450scc mRNA 1.4-fold after 24 h incubation, while 3000 and 10 000 ng DDE ml⁻¹ led to 0.39- and 0.18-fold reductions, respectively. The stimulatory effect of cholera toxin on P450scc mRNA was blocked by 3000 and 10 000 ng DDE ml⁻¹. Cholera toxin increased P450scc mRNA 3.48-fold after 48 h incubation, while 100 and 1000 ng methoxychlor ml⁻¹ increased P450scc mRNA 1.79- and 3.0-fold, respectively, and further increased the stimulatory effect of cholera toxin 6.47- and 5.44-fold, respectively. The results of the present study indicate that DDE inhibits granulosa cell steroidogenesis by affecting cAMP production and P450scc gene expression. However, methoxychlor appears to inhibit steroidogenesis by a mechanism occurring before the conversion of cholesterol into pregnenolone.

Introduction

Dichlorodiphenyltrichloroethane (DDT) is an insecticide that has been widely used around the world since the 1940s. By the early 1970s, it was discovered that intensive use of DDT resulted in bioaccumulation in the food chain and toxicity to wildlife, and as a result DDT was banned in the USA, Canada and several European countries. Nevertheless, DDT is still used extensively in other parts of the world as it is a very efficient and economic method for controlling malaria vectors and a variety of other insects that affect human health and agriculture (Lopez-Carrillo *et al.*, 1997; Rivero-Rodriguez *et al.*, 1997). Consequently, DDT and its most stable metabolite, *p,p*-dichlorodiphenyldichloroethylene (DDE), are continuously entering the

environment. These compounds have a tendency to concentrate in cold regions and have been found contaminating humans and wildlife in the Arctic regions and many other parts of the world (Ayotte *et al.*, 1995). In humans, DDE has been detected in blood, adipose tissue (Morgan and Roan, 1971) and ovarian follicular fluid (Jarrell *et al.*, 1993).

One of the most notable features of DDE is its ability to alter endocrine functions, resulting in its classification as an endocrine disrupter. Endocrine disruption by DDE was described in alligators exposed to a spill containing pesticides and other contaminants. The high concentrations of DDE found in the spill were believed to be responsible for altering steroid hormone concentrations and inducing phenotype abnormalities in alligators (Guillette Jr. *et al.*, 1995, 1996). These observations raised concerns about the long-term effect of DDE on human health. The presence of the pesticide in follicular fluid means that the oocyte and the granulosa cells that

*Correspondence and reprint requests.
Email: chedresj@duke.usask.ca

surround the oocyte are exposed to a potent endocrine disrupter during crucial stages of development and differentiation (Jarrell *et al.*, 1993). Thus, DDE might affect the viability of the oocyte and the endocrinology of the ovulatory follicle, with deleterious consequences for embryo health and the pregnancy. Low concentrations of DDE, similar to those that have been reported in the follicular fluid of women, may alter progesterone synthesis in granulosa cells (Crellin *et al.*, 1999), indicating that DDE affects ovarian function by altering steroid hormone synthesis.

Methoxychlor, a more labile and readily degradable compound, has been used to replace the banned DDT. However, methoxychlor is also an endocrine disrupter with anti-fertility effects described in a number of experimental models. In rats, treatment with methoxychlor induced a decrease in serum progesterone and impaired embryo implantation (Cummings and Laskey, 1993). Methoxychlor also induced abnormal oestrus in mice after neonatal exposure and caused a number of oestrogenic effects, including vaginal cornification and increased uterine mass (Eroschenko, 1991; Eroschenko *et al.*, 1995). In amphibians, methoxychlor inhibited progesterone-induced oocyte maturation (Pickford and Morris, 1999). Although methoxychlor does not persist in the environment, its continuous use exposes people and animals directly to amounts that have biological effects.

Collectively, this information indicates that both DDE and methoxychlor are potent endocrine disrupters, with the potential to affect human and animal reproduction adversely. The present investigation is a detailed study on the effects of DDE and methoxychlor on a stable steroidogenic pig granulosa cell model, JC-410, developed in our laboratory and validated for toxicological studies (Chedrese *et al.*, 1998; Crellin *et al.*, 1999). In this study, information is provided on the mechanisms by which DDE and methoxychlor affect ovarian steroidogenesis.

Materials and Methods

Reagents

Media M199, trypsin, penicillin, streptomycin and newborn calf serum were purchased from Gibco (Burlington, ON). Glutamine, cholera toxin, insulin, cAMP and materials for cAMP acetylation and purification, and 3-isobutyl-1-methyl-xanthine (IBMX) were purchased from Sigma (St Louis, MO). Plastic culture plates were purchased from Falcon (Lincoln Park, NJ). Iodinated progesterone (11- α -hydroxyprogesterone-11- β -D-glucuronide-[¹²⁵I]-iodotriamine) was purchased from Amersham Pharmacia Biotech (Piscataway, NJ). The Bio-Rad DC protein assay kit was purchased from Bio-Rad Laboratories (Hercules, CA). The DDE used in this study was purchased from ChemService (West Chester, PA) as pure *p,p*-DDE and *o,p*-DDE. The two DDE isoforms were combined so that the final preparation would contain 96% *p,p*-DDE and

4% *o,p*-DDE, dissolved in ethanol. These isoforms were mixed to represent environmental exposure. Methoxychlor (99.0%) (PolyScience Niles, IL) was dissolved in benzene as a 1% solution.

Cell cultures

Cells were grown in phenol red-free media 199 supplemented with 5% new born calf serum, 5 μ g insulin ml^{-1} , 100 iu penicillin ml^{-1} , 100 μ g streptomycin ml^{-1} , 2.2 g sodium bicarbonate l^{-1} and 4 mmol glutamine l^{-1} . Cultures were maintained in an incubator (Forma Scientific Inc., Marietta, OH) at 37°C in a water-saturated atmosphere of 5% CO₂ and 95% air. Experiments were carried out in 24-well culture plates. Once 70% confluency was attained, media were replaced with serum-free media containing the treatments. Times of exposure to DDE and methoxychlor, 24 and 48 h respectively, were determined in preliminary experiments. Vehicle for the DDE experiments consisted of 0.5% ethanol. The vehicle control for the methoxychlor experiments consisted of benzene in ethanol, at 0.01% benzene and 0.49% ethanol. Percentages given are v/v.

Quantification of progesterone, cAMP and cellular protein

Progesterone content was determined by radioimmunoassay in 100 μ l culture medium, as previously described by Chedrese *et al.* (1998). The inter- and intra-assay coefficients of variation for the progesterone assay were < 10%, and the minimum detectable content was 6.25 pg. Generation of cAMP was tested in cells exposed to the control medium or 100 ng cholera toxin ml^{-1} in the presence of the phosphodiesterase inhibitor IBMX (125 μ mol l^{-1}) for 30 min. Cellular cAMP was extracted with 100 μ l 1 mol HCl l^{-1} for 5 min and diluted with 900 μ l 0.05 mol sodium acetate buffer l^{-1} , pH 6.2, and quantified by radioimmunoassay (Chedrese *et al.*, 1998). The inter- and intra-assay coefficients of variation for the cAMP assay were < 15% and the minimum detectable content was 3.25 fmol. For protein content determination, cells were washed extensively with PBS and then solubilized with 200 μ l 0.1% SDS. Protein content was determined using the Bio-Rad DC protein kit.

Northern blot analyses

Total RNA was isolated by acid phenol:chloroform extraction according to the method described by Chomczynski and Sacchi (1987). Samples of total RNA were denatured, size-fractionated by electrophoresis on a 1% agarose-formaldehyde gel and transferred onto a nylon membrane by diffusion blotting. Pig P450sc (Mulheron *et al.*, 1989) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Tso *et al.*, 1985) cDNAs were used as probes. Probes were labelled by primer extension (Feinberg and Vogelstein, 1983) with [α -³²P] dCTP (> 3000 Ci mmol^{-1} ;

Fig. 1. Effect of dichlorodiphenyldichloroethylene (DDE) on cholera toxin-stimulated progesterone synthesis. Cells were cultured with 300–10 000 ng DDE ml⁻¹ and with or without 100 ng cholera toxin ml⁻¹. After 24 h incubation, culture media and cells were collected for progesterone and protein assays, respectively. Data are the mean $\pm$ SEM of three independent replications, expressed as fold values versus control (2.6 pg μ g⁻¹ protein). □, DDE; ■, DDE + cholera toxin. Values with different letters are significantly different ($P < 0.05$).

New England Nuclear, Boston, MA) to a specific activity of $1.5\text{--}3.0 \times 10^9$ d.p.m. mg⁻¹ DNA. Membranes were hybridized and autoradiography was performed as described by Chedrese *et al.* (1990), using the QuikHyb hybridization solution (Stratagene, La Jolla, CA). A Kodak Electrophoresis Documentation and Analysis System 120 was used for gel photography and densitometric analysis of autoradiographs. Each experiment was repeated three times.

Statistical analysis

Data are presented as the mean $\pm$ SEM of at least three independent experiments and were analysed using a two-way ANOVA (Figs 1–5). When a significant F value was present, Fisher's least significant difference test was used for individual comparison of means (Steel and Torrie, 1980). Significant differences, indicated by different letters, were defined as $P < 0.05$. Fold values were calculated by dividing all data by the mean of the control group.

Results

Progesterone synthesis was increased 1.7-fold by 100 ng cholera toxin ml⁻¹ and decreased 0.49- and 0.25-fold by 3000 and 10 000 ng DDE ml⁻¹, respectively, after 24 h incubation. The stimulatory effect of cholera toxin on progesterone synthesis was blocked by 300–10 000 ng DDE ml⁻¹ (Fig. 1). Progesterone synthesis was not affected

by methoxychlor at concentrations ranging from 1 to 100 ng ml⁻¹, after 48 h incubation. However, a higher concentration of methoxychlor, 1000 ng ml⁻¹, decreased progesterone synthesis 0.32-fold. The cholera toxin-stimulated progesterone synthesis was blocked by 100 and 1000 ng methoxychlor ml⁻¹ (Fig. 2).

Synthesis of cAMP was stimulated 7.2-fold by 100 ng cholera toxin ml⁻¹. Conversely, 3000 and 10 000 ng DDE ml⁻¹ decreased cAMP synthesis 0.66- and 0.36-fold, respectively. At 300, 3000, and 10 000 ng ml⁻¹ DDE also decreased the cholera toxin-stimulated cAMP synthesis 0.84-, 0.68- and 0.52-fold, respectively (Fig. 3). At 1–100 ng ml⁻¹ methoxychlor did not affect basal or cholera toxin-stimulated cAMP synthesis (Fig. 4).

Protein concentrations were not altered by 100 ng cholera toxin ml⁻¹ or 300 and 1000 ng DDE ml⁻¹. However, at both the 3000 and 10 000 ng DDE ml⁻¹, protein concentrations were increased 1.35- and 1.42-fold, respectively, in either the presence or absence of cholera toxin (Fig. 5). Protein concentration, which correlates with the number of cells, was not altered by 1–100 ng methoxychlor ml⁻¹, in the presence or absence of cholera toxin (data not shown).

P450scc mRNA was increased 1.4-fold by 100 ng cholera toxin ml⁻¹ after 24 h incubation, while 3000 and 10 000 ng DDE ml⁻¹ decreased P450scc mRNA to 0.39- and 0.18-fold, respectively. The cholera toxin-stimulated amounts of P450scc mRNA were blocked by 3000 and 10 000 ng DDE ml⁻¹ (Fig. 6). At 100 ng cholera toxin ml⁻¹,

Fig. 2. Effect of methoxychlor (MXC) on cholera toxin-stimulated progesterone synthesis. Cells were cultured with 1–1000 ng methoxychlor ml⁻¹ and with or without 100 ng cholera toxin ml⁻¹. After 24 h incubation, culture media and cells were collected for progesterone and protein assays, respectively. Data are the mean $\pm$ SEM of three independent replications, expressed as fold values versus control (8.1 pg μ g⁻¹ protein). $\square$, MXC; $\blacksquare$, MXC + cholera toxin. Values with different letters are significantly different ($P < 0.05$).

Fig. 3. Effect of dichlorodiphenyldichloroethylene (DDE) on cholera toxin-stimulated cAMP synthesis. Cells were cultured with 300–10 000 ng DDE ml⁻¹ for 24 h. Culture media was then replaced with culture media containing 125 μ mol 3-isobutyl-1-methyl-xanthine (IBMX) l⁻¹ and with or without 100 ng cholera toxin ml⁻¹. After 30 min incubation, cells were collected for cAMP assay. Data are the mean $\pm$ SEM of three independent replications, expressed as fold values versus control (50.05 fmol μ g⁻¹ protein). $\square$, DDE; $\blacksquare$, DDE + cholera toxin. Values with different letters are significantly different ($P < 0.05$).

Fig. 4. Effect of methoxychlor (MXC) on cholera toxin-stimulated cAMP synthesis. Cells were cultured with 1–1000 ng methoxychlor ml⁻¹ for 48 h. Culture media were then replaced with culture media containing 125 μ mol 3-isobutyl-1-methyl-xanthine (IBMX) l⁻¹ with or without 100 ng cholera toxin ml⁻¹. After 30 min incubation, cells were collected for cAMP assay. Data are the mean $\pm$ SEM of three independent replications, expressed as fold values versus control (32.2 fmol μ g⁻¹ protein). $\square$, MXC; $\blacksquare$, MXC + 100 ng cholera toxin ml⁻¹. Values with different letters are significantly different ($P < 0.05$).

Fig. 5. Effect of dichlorodiphenyldichloroethylene (DDE) on protein content. Cells were cultured with 300–10 000 ng DDE ml⁻¹. After 24 h incubation, cells were collected for protein assay. Data are the mean $\pm$ SEM of three independent replications, expressed as fold values versus control. $\square$, DDE; $\blacksquare$, DDE + cholera toxin. Values with asterisks are significantly different from ($P < 0.05$) controls (30.8 μ g).

Fig. 6. Effect of dichlorodiphenyldichloroethylene (DDE) on cholera toxin (CT)-stimulated P450scc mRNA and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA concentrations. Cells were cultured with 3000 or 10000 ng DDE ml⁻¹ and with or without 100 ng cholera toxin ml⁻¹. After 24 h incubation, cells were collected for northern blot analysis. (a) Autoradiograph of a northern blot from a representative experiment. (b) Graph of the densitometric analysis data, expressed as fold values versus control. □, DDE; ■, DDE + cholera toxin.

P450scc mRNA was increased 3.48-fold after 48 h incubation. At 100 and 1000 ng ml⁻¹, methoxychlor also increased P450scc mRNA 1.79- and 3.0-fold, and further increased cholera toxin-stimulated amounts of P450scc mRNA 6.47- and 5.44-fold, respectively (Fig. 7).

Discussion

The present study investigated the effect of DDE and methoxychlor on progesterone synthesis on a stable steroidogenic pig granulosa cell line, JC-410. The cells of this line do not respond to the gonadotrophins FSH and LH, the physiological regulators of granulosa cell steroidogenesis. However, they do respond to cholera toxin, a protein kinase A (PKA) activator that mimics the effect of FSH on progesterone synthesis in granulosa cells.

Previous investigations have shown that low concentrations of DDE (10–100 ng ml⁻¹) increase PKA-stimulated progesterone synthesis after 24 h incubation (Crellin *et al.*, 1999). This effect is mediated by an increase in the expression of the P450scc gene, which is considered to be a regulated step in progesterone synthesis

Fig. 7. Effect of methoxychlor (MXC) on cholera toxin (CT)-stimulated P450scc mRNA and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA concentrations. Cells were cultured with 100 or 1000 ng methoxychlor ml⁻¹ and with or without 100 ng cholera toxin ml⁻¹. After 24 h incubation, cells were collected for northern blot analysis. (a) Autoradiograph of a northern blot from a representative experiment. (b) Graph of the densitometric analysis data, expressed as fold values versus control. □, MXC; ■, MXC + cholera toxin.

(Richards, 1994). The results of the present study demonstrated that high concentrations of DDE (1000–10000 ng ml⁻¹) decreased basal and cholera toxin-stimulated progesterone synthesis and that DDE had a similar inhibitory effect on basal and cholera toxin-stimulated cAMP generation. As the effect of cholera toxin on P450scc mRNA was completely blocked, while cAMP generation was decreased only to a small extent, it is possible that high concentrations of DDE also directly affect P450scc gene expression. Thus, the decrease in the expression of the P450scc gene is the consequence of the inhibitory effect of DDE on cAMP generation. From these results it is possible to conclude that DDE affects progesterone synthesis in granulosa cells through two different mechanisms. The first mechanism, observed with low concentrations of DDE, potentiates the effect of PKA activators on the expression of P450scc, resulting in an increase in the conversion of cholesterol to pregnenolone. The second mechanism, observed with high concentrations of DDE, impairs generation of cAMP.

Exposure to DDE also increased granulosa cell protein synthesis, an effect that was independent of PKA stimulation. In contrast, methoxychlor alone or in combination with cholera toxin did not affect protein synthesis. DDE has been described as a weak oestrogen,

capable of binding and activating the oestrogen receptor (Coldham *et al.*, 1997; Andersen *et al.*, 1999) and also as an anti-androgen (Kelce *et al.*, 1995). Although the presence of androgen receptors has not been demonstrated definitively in the JC-410 cells, secondary experimental evidence indicates that it is present (Rodway *et al.*, 1999). It is also known that endogenous androgen or oestrogen synthesis does not occur in these cells. Thus, if androgens do stimulate cell proliferation, this may occur after their conversion into oestrogens, since JC-410 cells have an active aromatase system. Therefore, it is possible that

the increase in protein synthesis, observed in the present study, was the result of increased cellular proliferation induced by DDE. This result is in accordance with the reported oestrogenic effects of DDE observed in the oestrogen-responsive MCF-7 breast cancer cells (Soto *et al.*, 1995). Hence, the inhibitory effect of DDE on steroidogenesis may be dissociated from its proliferative actions.

Although it is accepted that methoxychlor has antifertility effects, its mechanism of action has not yet been elucidated. In the present study, exposure to methoxychlor for 48 h inhibited progesterone synthesis in granulosa cells and this inhibitory effect is consistent with the observation that methoxychlor decreased serum progesterone concentrations in pregnant rats (Cummings and Laskey, 1993). Methoxychlor may affect progesterone synthesis by blocking a step in the steroidogenic pathway distal to cAMP generation, but before pregnenolone synthesis. Granulosa cells are surrounded by a basal membrane, which limits the entrance of lipoproteins into the follicular cavity. Consequently, cholesterol is mainly obtained from acetate in a reaction catalysed by HMG-CoA reductase, which is a crucial step in granulosa cell steroidogenesis (Richards, 1994). The expression and activity of HMG CoA reductase in granulosa cells is under the control of FSH and oestradiol (Maitra *et al.*, 1995; Di Croce *et al.*, 1999). Steroid hormone synthesis also depends on the transport of cholesterol into the mitochondria, which is mediated by steroid acute regulatory protein (StAR) (Stocco and Clark, 1996). Therefore, it is possible that HMG-CoA reductase or StAR, or both, are potential targets for the endocrine disrupting activity of methoxychlor.

The effects of the gonadotrophins are mediated through mechanisms involving synthesis of cAMP and activation of PKA, which trigger a cascade of intracellular reactions leading to steroid synthesis. Concentrations of DDE or methoxychlor sufficient to block PKA-stimulated progesterone synthesis could reduce the effect of FSH by inhibiting its signal cascade and the steroidogenic pathway. FSH is crucial for the normal development of the ovarian follicle and for oocyte maturation. Therefore, an inhibitory effect of DDE and methoxychlor on FSH-induced steroidogenesis may result in a subtle but detrimental effect on granulosa cells, causing a higher number of anovulatory

cycles and, consequently, affecting fertility.

The low concentrations of DDE used in a previous study (Crellin *et al.*, 1999), which increased PKA-stimulated progesterone synthesis, were in the range of concentrations reported to be contaminating human follicular fluid in a Canadian study (Jarrell *et al.*, 1993). The higher concentrations of DDE used in the present study are in the range of the serum concentrations detected in populations exposed to high amounts of DDT (Lopez-Carrillo *et al.*, 1997; Rivero-Rodriguez *et al.*, 1997). Methoxychlor, although more degradable in the environment, may also cause toxicity by direct exposure. It is quite possible that differences in the potencies of DDE and methoxychlor may contribute to the different mechanisms of action in granulosa cells. The results of the present study indicate that both DDE and methoxychlor inhibit basal- and stimulated-progesterone synthesis in granulosa cells and that the normal effect of gonadotrophins may be hindered in individuals as a consequence of exposure to DDE or methoxychlor. Thus, both the presence of DDE in the environment and the use of methoxychlor may present a threat to the health and fertility of humans and domestic and wild animals.

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