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Cadmium interferes with steroid biosynthesis in rat granulosa and luteal cells *in vitro*

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Recently, cadmium has been described to disturb ovarian function in rats. In this paper the direct influence of cadmium on steroid production of ovarian cells *in vitro* has been studied. Granulosa and luteal cells were obtained from proestrous and pregnant rats, and incubated with 0, 5, 10, 20 or 40 $\mu g\,ml^{-1}$ $CdCl_2$ in the presence or absence of 0.1–1000 $ng\,ml^{-1}$ follicle stimulating hormone (FSH) or luteinizing hormone (LH) for 24 or 48 h. Production of progesterone (P) and 17β-estradiol (E_2) by granulosa and that of P by luteal cells were measured by radioimmunoassay. In FSH-stimulated granulosa cell cultures, 5 and 40 $\mu g\,ml^{-1}$ $CdCl_2$ suppressed P accumulation to 65 and 10%, respectively; accumulation of E_2 (at 5 $\mu g\,ml^{-1}$ $CdCl_2$) decreased to 44%. P production of LH-supported luteal cells dropped to 86 and 66%, respectively, when 5 and 40 $\mu g\,ml^{-1}$ $CdCl_2$ was added to the medium. No alteration in basal P accumulation occurred in granulosa and luteal cell cultures following incubations with 20 and 40 $\mu g\,ml^{-1}$ $CdCl_2$, whereas basal E_2 production of granulosa cells was markedly diminished. It is concluded that $CdCl_2$ suppressing steroid synthesis *in vitro* exerts a direct influence on granulosa and luteal cell function.

Keywords: cadmium, estradiol production, granulosa cells, luteal cells, progesterone production, steroid biosynthesis

Introduction

The irreversible damage to testicles induced by cadmium in laboratory rodents is well documented (see Setchell 1978 for review, Koizumi & Waalkes 1988, Waalkes *et al.* 1988a,b). Recently, cadmium given to diestrous rats as a single s.c. injection has been shown to inhibit ovulation in 50% of animals by lowering the proestrous luteinizing hormone (LH) content in the pituitary and in the serum (Paksy *et al.* 1989). This adverse effect of cadmium proved to be reversible as it could be overcome by excess amounts of luteinizing hormone releasing hormone (LHRH) (Varga & Paksy 1991). This finding clearly demonstrates that cadmium influences ovarian function indirectly. A direct impact, however, cannot be excluded, as, in hamsters, cadmium has been reported to be sequestered by theca cells, while no accumulation in the granulosa layer or in the oocyte could be measured. Uptake of cadmium by corpora lutea of pregnant hamsters (Dencker 1975) as well as pseudopregnant (Paksy *et al.* 1990a) and cyclic rats (Varga *et al.* 1991) has also been shown.

The aim of the present work has been to investigate whether cadmium exerts a direct influence on the steroidogenic potential in non-stimulated and gonadotroph-stimulated rat granulosa and luteal cells *in vitro*.

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Materials and methods

Materials

Eagle's modified essential medium (EMEM), cat. no. 10-101-22, was obtained from Flow Laboratories (Irvine, UK), follicle stimulating hormone (pFSH) and collagenase (type VII) were purchased from Sigma (St Louis, MO), luteinizing hormone (pLH) was obtained from Calbiochem (San Diego, CA), testosterone was obtained from BDH (Poole, UK), $CdCl_2 \cdot 2.5\,H_2O$ and Trypan blue were purchased from Reanal (Budapest, Hungary), fetal calf serum (FCS) was obtained from Humán (Budapest,

Hungary), pentobarbital from Rohne Poulenc (Paris, France). Radioimmunoassay (RIA) progesterone (P) and 17β-estradiol (E_2) antisera were the generous gift of Dr G. D. Niswender (Fort Collins, CO). The 24-well tissue culture plates (TC sterile no. 662 160) were purchased from Greiner GmbH (Kremsmünster, Austria).

Animals

CFY rats (LATI, Gödöllö, Hungary) weighing 220–280 g were maintained under a lighting schedule of 12 h light/12 h dark (lights on: 7 a.m.). Vaginal smears were checked daily to determine the stage of the estrous cycle. Rats displaying at least three consecutive 4 day cycles were used.

Isolation and treatment of granulosa cells

Animals were anesthetized (40 mg kg^{-1} pentobarbital) in proestrus at 8–9 a.m. Uterine ballooning was checked, and ovaries were removed and cleaned. The largest (0.6–1.0 mm in diameter)—and due to start vascularization—slightly reddish preovulatory follicles were selected, counted and punctured by stainless steel needles in order to obtain the granulosa cells released into the chilled medium (EMEM) using gentle pressure and agitation of the theca capsule. Experiments were repeated at least 5 times (five or six rats, 8–10 follicles per rat, i.e. 40–60 follicles per experiment). Cells were collected by gentle centrifugation (800 r.p.m. for 10 min, 4 °C), resuspended in EMEM (washing), centrifuged and resuspended in EMEM containing 10% FCS. They were counted and diluted to 10^6 cells ml^{-1}. Viability was 80–85% as estimated by the Trypan blue exclusion test. The cell suspension was dispersed to 24-well culture plates, diluted to a final concentration of 7.5×10^5 cells ml^{-1}/well with EMEM containing 10% FCS and 0.5 μM testosterone. Cells were cultured in the presence or absence of FSH (0.1–1000 ng ml^{-1}) and with or without $CdCl_2$ (5–40 μg ml^{-1}) in duplicates for 48 h at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. At the end of exposure media were collected, centrifuged (2000 r.p.m., 10 min, 4 °C) and frozen (−20 °C) for later steroid analysis. FSH and $CdCl_2$ were dissolved in EMEM without FCS. Testosterone (0.5 μM = 144 ng ml^{-1}) was dissolved in ethanol at a concentration of 43.2 μg ml^{-1} (Fortune & Hilbert 1986).

Isolation and treatments of luteal cells

Proestrous rats were caged overnight with fertile males, the next morning was designated as day 1 of pregnancy if sperms were found in the vaginal lavage. Ovaries of rats on day 5 of pregnancy were dissected under pentobarbital anesthesia, and corpora lutea were removed, cleaned and cut into four pieces in a Petri dish containing 5 ml of chilled dispersion medium (EMEM) with 1% FCS and 200 IU collagenase ml^{-1}. This first fraction of dispersion medium was discarded and replaced with fresh medium. Then, luteal tissue was placed in a shaking water bath incubator for five consecutive 15 min incubations with fresh collagenase under an atmosphere of 95% air and 5% CO_2 at 37 °C.

The fractions of dispersing media containing loosened cells were pooled at 4 °C. Disintegration of luteal tissue was repeatedly promoted by gently drawing it through a pipet. The pooled fractions were filtered through two layers of a nylon mesh to discard undispersed tissue remnants. The cell suspension was washed 3 times by centrifugation (2000 r.p.m., 10 min, 4 °C) and resuspended in chilled EMEM. Cell viability proved to be 70–80%. Cells were recovered from luteal tissue of six to eight rats in each experiment repeated at least 5 times. Cells were cultured for 24 h in duplicate under similar circumstances described for granulosa cells, plated at a concentration of 7.5×10^5 cells ml^{-1}/well with or without LH and/or $CdCl_2$. Spent media for P determination were stored as those of granulosa cells.

Statistical evaluation

Analysis of variance and Dunnett's test were used for statistical evaluations.

Results

Granulosa cells

P accumulation. During the 48 h incubation period, P production of unstimulated cells was 5.309 (±2.18) ng ml^{-1} (± SE, $n = 6$), which was considered as the baseline level of P accumulation. This level (100%) increased 3- to 5-fold in FSH supported cultures. $CdCl_2$ on its own had no marked influence on basal P secretion, whereas when combined with FSH it significantly suppressed P accumulation (Figure 1).

E_2 accumulation. Basal (100%) E_2 secretion of granulosa cells cultured with testosterone for 48 h was 1311 (± 132) pg ml^{-1} (± SE, $n = 6$). No significant rise in E_2 accumulation could be detected under the influence of FSH (0.1–1000 ng ml). Basal E_2 production decreased significantly to 58 and 42%, respectively, at $CdCl_2$ concentrations of 20 and 40 μg ml^{-1}. E_2 production stimulated by FSH (1 ng/ml) was suppressed by $CdCl_2$ (5 μg ml^{-1}). At a higher FSH level only the highest $CdCl_2$ concentration induced a marked suppression of E_2 accumulation (Figure 2).

Luteal cells

The baseline (100%) P production of luteal cells proved to be 5.949 (± 0.62) ng ml^{-1} (± SE, $n = 12$). LH (10 and 100 ng ml^{-1}) increased P accumulation in a dose-dependent manner. Under the effect of $CdCl_2$, the basal P production of luteal cells re-

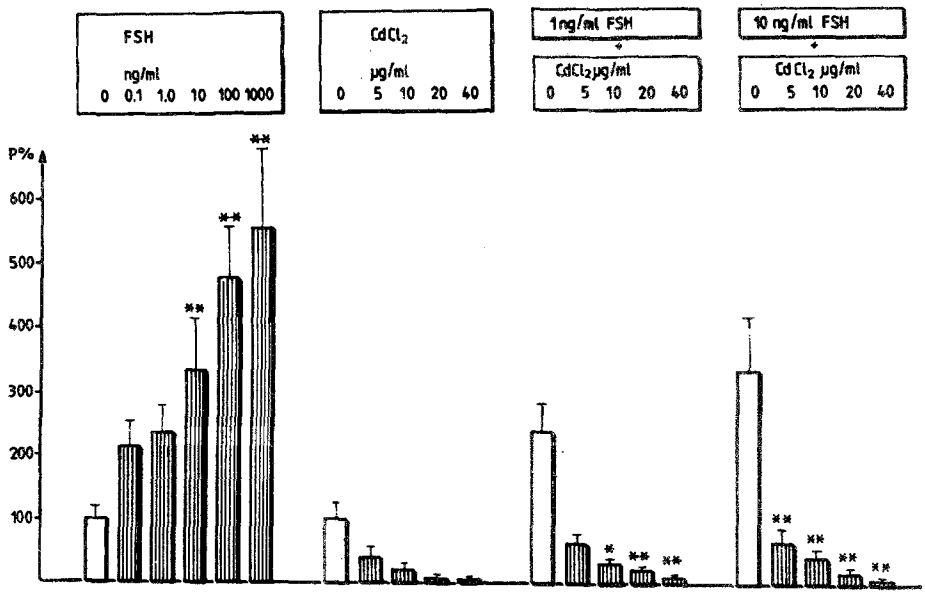

Figure 1. Effect of $CdCl_2$ on basal ($n = 6$) and FSH-induced ($n = 6$) P production *in vitro* on granulosa cells obtained from follicles of proestrous rats. Data are expressed as percent of baseline P production of 7.5×10^5 cells (5.309 ± 2.18 ng ml^{-1}) over 48 h. Statistically significant differences from baseline are indicated: $^*P < 0.05$; $^{**}P < 0.01$.

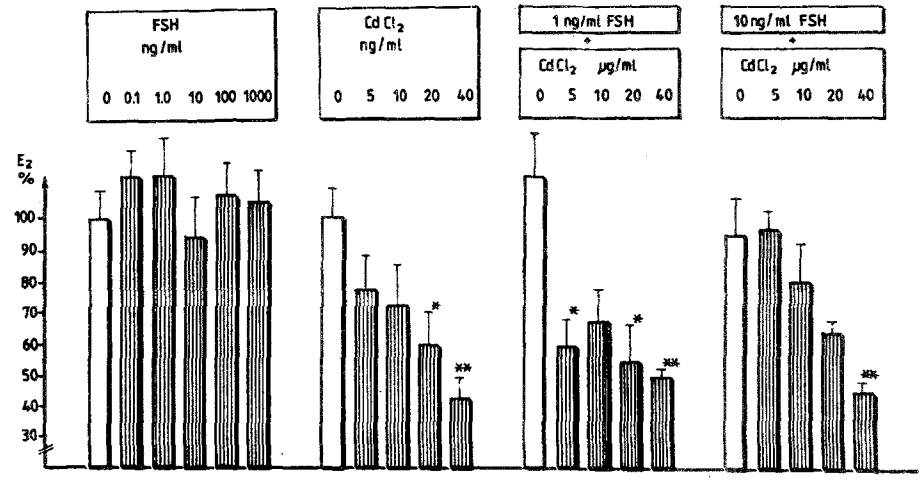

Figure 2. Effect of $CdCl_2$ on basal ($n = 6$) and FSH-induced ($n = 6$) E_2 production *in vitro* on granulosa cells obtained from follicles of proestrous rats. Data are expressed as percent of baseline P production of 7.5×10^5 cells (1311 ± 132.5 pg ml^{-1}) over 48 h. Statistically significant differences from baseline are indicated: $^*P < 0.05$; $^{**}P < 0.01$.

mained unchanged, whereas the LH-induced rise in P accumulation was markedly decreased (Figure 3).

Discussion

Cadmium exerted an inhibitory action on the steroidogenesis of granulosa and luteal cells *in vitro*. The suppression of steroid synthesis depended on the concentration of $CdCl_2$ and on the level of gonadotrophic stimulation.

Cadmium in the human ovary has been reported to accumulate with age and is related to smoking habits (Zsolnai *et al.* 1992). The extremely high individual cadmium levels of 0.494 µg g^{-1} Cd wet weight found among non-smokers (mean: 0.115 ± 0.012 µg g^{-1} Cd; $n = 52$) and 0.512 µg g^{-1} Cd wet weight found among smokers (mean: 0.149 ± 0.020 µg g^{-1} Cd; $n = 29$) in ovarian tissues are only one order of magnitude lower than the lowest effective cadmium concentration applied in our *in vitro* experiment, i.e. 5 µg ml^{-1}.

The avascular granulosa cell layer in the follicle *in vitro* seems to be protected against blood chemicals. Following a s.c. injection, granulosa cells in the

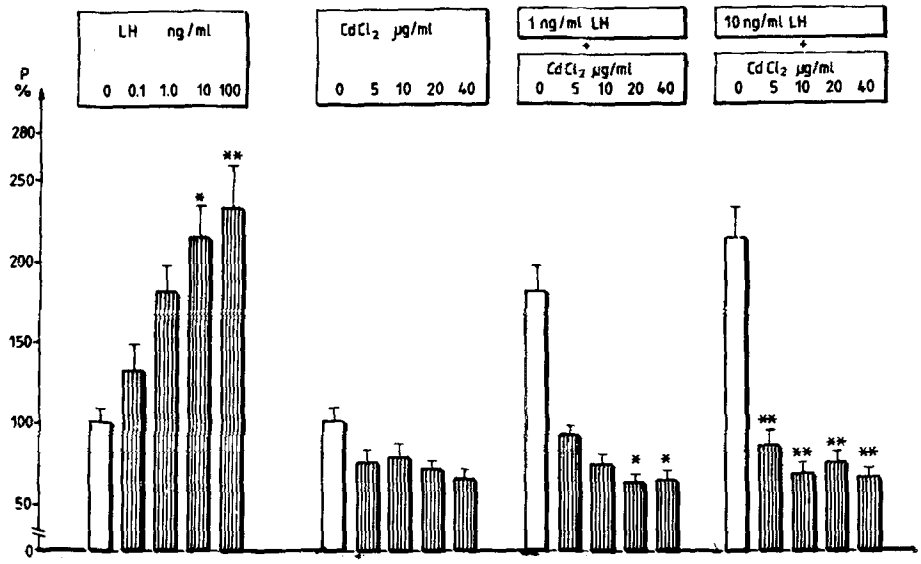

Figure 3. Effect of $CdCl_2$ on basal ($n = 12$) and LH-induced ($n = 6$) P production *in vitro* on luteal cells obtained from corpora lutea of day 5 pregnant rats. Data are expressed as percent of baseline P production of 7.5×10^5 cells (5.949 ± 0.62 ng ml^{-1}) over 24 h. Statistically significant differences from baseline are indicated: $^*P < 0.05$; $^{**}P < 0.01$.

ovaries of Golden hamsters do not accumulate cadmium, unlike the thecal cells (Dencker 1975).

Cadmium entering the circulation is first bound to mercaptalbumin (Suzuki *et al.* 1986). The blood–follicle barrier may slow down the route of cadmium to reach granulosa cells: (i) less than 100% of plasma proteins are found in follicular fluid, (ii) higher molecular weight proteins like albumins penetrate more slowly than small ones, (iii) follicular swelling is sluggish (Gosden *et al.* 1988) and (iv) circulating cadmium, freed from the low stability constant protein complex, soon forms a less toxic, stable biocomplex with metallothionein (Scheuhammer *et al.* 1985, Sumi *et al.* 1987).

Nevertheless, as cadmium has been reported to accumulate in the theca layer of the ovary in Golden hamsters (Dencker 1975) as well as in non-luteal ovarian tissue of pseudopregnant rats (Paksy *et al.* 1990a), the possibility that cadmium may reach the avascular granulosa layer by diffusion or/and via the thecal blood, and that thecal cadmium alone is able to disturb steroid biosynthesis, cannot be ruled out. Ovulatory disturbances following a $CdCl_2$ injection lasting about 12 cycles before spontaneous recovery commenced have been reported (Paksy *et al.* 1990b).

In the present experiments, cadmium exerted a direct effect on luteal cell function, i.e. lowering P production *in vitro*. This is in good accordance with our earlier *in vivo* findings where a direct influence of cadmium on luteal function was found to be likely because (i) following a s.c. injection on day 1 of pseudopregnancy, corpora lutea do incorporate cadmium at a higher rate than non-luteal tissue, pituitary and adrenals, and (ii) a decrease in serum P levels during pseudopregnancy caused premature luteal regression in pseudopregnant rats (Paksy *et al.* 1990a), moreover a decreased ovarian P secretion rate in early pregnancy of rats was described (Paksy *et al.* 1992).

Thus, unlike granulosa cells, luteal cells which have ready access to general circulation are rather unprotected against blood chemicals. Due to the specific high vascularity, the elevated luteal blood flow (Janson *et al.* 1981, Varga *et al.* 1985) and the high permeability of capillaries to plasma proteins in corpora lutea (Morriss & Sass 1966), the entry of cadmium into luteal tissue can take place to a greater extent. The mechanism of cellular uptake and action of cadmium is unknown. It is supposed to bind to an SH group-containing membrane receptor and then be transported to the cell interior by an active transport mechanism, probably endocytosis (Klug *et al.* 1988). The major route of Cd^{2+} uptake has been reported through voltage-gated dihydropyridine-sensitive Ca^{2+} channels. The entry of $^{109}Cd^{2+}$ through the channels, although low relative to Ca^{2+} (0.3% of Ca), is critical in cadmium toxicity; moreover, Cd^{2+} is a potent blocker of depolarization-stimulated $^{45}Ca^{2+}$ uptake (Hinkle *et al.* 1987). Cadmium is able to mobilize stored cellular calcium by stimulating a cell surface 'receptor' and by increasing inositol triphosphate (Smith *et al.* 1989).

Inhibition of hormone biosynthesis may partly

involve the ability of cadmium to compete with calcium.

In selectively calcium-deficient incubation the capacity of 8-bromo-cAMP to stimulate pregnenolon synthesis from endogenous substrate is significantly impeded (Veldhuis *et al.* 1984).

With regard to our present experiments, cadmium is considered to enter granulosa cells, as basal E_2 secretion was dose-dependently decreased in cadmium treated cells. Considering that basal P secretion was not influenced, the possible interference of cadmium with the activity of the aromatase system cannot be excluded.

However, the finding that basal P production of cadmium-treated cells remained unchanged and that only the gonadotroph-stimulated response is inhibited in granulosa as well as in luteal cells supports the theory of cadmium–calcium competition. Calcium has been reported not to influence basal pregnenolon production or estrogen synthesis from androgen substrates, or P production from exogenously supplied sterol substrates in swine granulosa cells, whereas it is known that Ca^{2+} ions influence the stimulatory actions of LH and modulate the effects of cAMP on P biosynthesis (Veldhuis *et al.* 1984). The calcium ionophore A 23187 has no effect on P synthesis at any stage of the cycle; however, it enhances the ability of LH to increase P synthesis in luteal cell preparations (Hansel & Dowd 1986).

Cadmium may modulate LH action in ovarian granulosa cells, in part, by calcium calmodulin-dependent mechanisms. It binds to all the Ca^{2+} binding sites of calmodulin and induces as considerable a conformational change in calmodulin as Ca^{2+} (Akiyama *et al.* 1990). Cadmium is able to substitute for calcium in activating calmodulin-dependent cAMP phosphodiesterase (Nimura *et al.* 1987) and has been reported to cause disassembly of the cytoplasmic microtubule complex (Perrino, 1986). LH receptors on granulosa cells are located over the microvilli (actin-rich filaments) area at the cell circumference. The receptor molecule has been suggested to be associated with contractile cytoskeletal elements playing a role in receptor mobility (Amsterdam & Rotmensch 1987). E_2 production in granulosa cells could not be stimulated by FSH. Our experiments were carried out in serum containing medium and such serum may prevent FSH stimulating steroidogenesis in the granulosa cells (Orly *et al.* 1980).

In summary, apart from the recent evidence (Paksy *et al.* 1989, 1990a) that cadmium is a potent female reproductive toxicant in rats, exerting its action mainly at the level of the pituitary and ovary, present data demonstrate that cadmium is able to interfere directly with hormone production in the steroid producing cell.

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