

IN VITRO EFFECT OF TRIIODOTHYRONINE ON THE CYCLIC AMP, PROGESTERONE AND TESTOSTERONE LEVEL IN PORCINE THECA, GRANULOSA AND LUTEAL CELLS

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Objective. To investigate the influence of thyroid hormone on steroid production and cAMP accumulation in porcine theca (Tc) and granulosa cells (Gc) isolated from preovulatory follicles as well as in luteal cells isolated during the mid-developing luteal phase.

Methods. Granulosa and theca cells separated from pig ovarian follicles and pieces of corpora lutea were cultured in the incubation medium M199 with 5 % calf serum. After the addition of triiodothyronine (T_3) and 3-isobutyl-1-methyl xanthine (IBMX) to the culture medium cAMP, progesterone and testosterone were estimated with the aid of specific RIA.

Results. T_3 added to the culture media stimulated the steroid secretion and cAMP accumulation in all cell types investigated. In theca cells, T_3 alone increased androgen production by 2 fold and the addition of IBMX further augmented the steroidogenesis by 2.2 fold. In granulosa cell culture, IBMX had no effect either on the basal or T_3 stimulated progesterone secretion and cAMP accumulation. In luteal cell culture, IBMX added alone increased progesterone secretion and cAMP accumulation in the same manner as T_3 . Further augmentation of progesterone secretion (1.3-fold) and cAMP accumulation (1.1-fold) was observed after the addition of IBMX together with T_3 .

Conclusion. The influence of thyroid hormone on cyclic nucleotide release by ovarian cells may suggest the involvement of cAMP-dependent mechanism in the realization of T_3 action in ovarian cells.

Keys words: Triiodothyronine – cAMP accumulation – Progesterone – Testosterone – Theca, granulosa and luteal cells – Pig

CHANNING et al. (1976) reported that the combination of thyroid hormone, insulin and cortisol enhanced LH and FSH induced luteinization of porcine granulosa cells cultured *in vitro*. In porcine granulosa cells WAKIM et al. (1987) found nuclear binding sites for T_3 and which were later (WAKIM et al. 1994) defined as specific functional thyroid receptors (TR- α and TR- β) in human granulosa cells. BHATTACHARYA et al. (1988) showed the presence of thyroid hormone binding sites in the human corpus luteum and GOLDMAN et al. (1993) demonstrated that human luteinized granulosa cells contain thyroid

hormone binding sites and that these cells serve as target for T_3 action in particular T_3 modulation of hCG-mediated ovarian cell proliferation and function.

Recently GREGORASZCZUK and SKALKA (1996) observed that the addition of T_3 to the culture medium increased androgen secretion by theca interna cells (Tc) isolated from porcine preovulatory follicles with a simultaneous decrease of estradiol secretion by granulosa cells (Gc) cultured both alone and in co-culture with Tc. T_3 also stimulated progesterone secretion by luteal cells isolated from early-develop-

ing and mid-developing corpora lutea (GREGORASZCZUK 1996) suggesting a direct action on Tc and luteal cells. To characterize the nature of thyroid hormone action in the ovary before and after ovulation, the direct effects of T_3 on steroid secretion and cAMP accumulation were investigated *in vitro* using a culture system of porcine Tc and Gc isolated from large preovulatory follicles and luteal cells collected from mid-developing corpora lutea.

The increase of cAMP accumulation could be due by enhanced cAMP synthesis and/or decrease of cAMP degradation. The participation of the inhibition of cAMP degradation in total increase of cAMP accumulation was assessed by the addition of 3-isobutyl-1-methyl xanthine (IBMX) an inhibitor of phosphodiesterase (PDE) to the culture medium.

Material and Methods

Cell isolation and culture. Pig ovaries were obtained from slaughterhouse animals and classified according to AKINS and MORRISSETTE (1968) and CHANNING and LEDWITZ-RIGBY (1975). Only proestrus ovaries containing large vascular follicles and corpora lutea albicantia were selected as the source of theca cells (Thc) and granulosa cells (Gc). The separation of Gc from the theca layer was performed according to STOKLOSOWA (1978). The Gc were scraped from the follicular wall with round trip ophthalmologic tweezers and rinsed several times with PBS. Almost 100 % of the Gc were removed from the follicle, average yield of recovered cells being 1.3×10^6 cells per ml medium. After rinsing and centrifugation the cells were resuspended in the incubation medium M199 supplemented with 5 % calf serum. In the meantime, the theca layer from the same follicle was removed under a dissecting microscope, rinsed from entangled Gc, weighed and transferred to multiwell plates, the whole theca layer being used per well per 1 ml medium.

Corpora lutea excised from ovaries in the luteal phase (days 5-7 after ovulation) and deprived of their connective tissue capsule were cut into small pieces, weighed and transferred to multiwell plates. The phase of the luteal cells was determined according to SCHILLING (1974) and GREGORASZCZUK (1992). The cultures were maintained in the culture medium at 37 °C in humidified atmosphere of 5 % CO_2 in the

air. The media collected after the culture were frozen and stored for further steroid analysis.

Triiodothyronine (T_3) (Sigma, St. Louis, MO, USA) in a dose of 10^{-9} M and 3-isobutyl-1-methylxanthine (IBMX, Sigma, St. Louis, MO, USA) in a dose 0.1 mM were added to the wells of experimental group. The doses of T_3 and IBMX were chosen according to our previous experience (GREGORASZCZUK and SKALKA 1996) and from the literature (GOLDMAN et al. 1993).

cAMP and steroids assays. For the estimation of cAMP, the incubations were carried out in a serum-free medium with or without IBMX or 10^{-9} M T_3 . After 2 h incubation, cAMP accumulation in the cells was measured with commercial RIA kit (Amersham International, Amersham, UK; cAMP [3H] assays system code TRK 432). The coefficient of variation was less than 7 % over the range 0.5 – 14 pmol/50 ml sample). The cross reactivity of the antiserum used with cGMP was less than 0.01 %, with AMP 0.003 %, with ADP 0.003 % and with ATP less than 0.003 % (all compared to cAMP). Progesterone and testosterone concentration in the medium at the end of the culture period were estimated by radioimmunoassay described previously (STOKLOSOWA et al. 1982).

Progesterone assay. A highly specific antibody raised in sheep against 11α -hydroxy-progesterone hemisuccinate coupled to bovine serum albumin was used. The cross-reaction with pregnenolone was 2.9 %. All other tested steroids (cholesterol, 20β -dehydroprogesterone, testosterone, dehydroepiandrosterone, androsterone and estrone) showed less than 1 % cross-reaction. [$1,2,6,7\text{-}^3H$] progesterone (Radiochemical Centre, Amersham, England, sp. act. 80Ci/mmol) was used as the tracer. The limit of assay sensitivity was 50 pg/ml. The coefficients of variation within and between assays were 15 % and 2.5 % respectively.

Androgens assay. Antiserum was induced in rabbits against testosterone-3-9-carboxy-methyl-oxime-BSA as the antigen. The antisera exhibited 100 % binding of testosterone and cross-reacted 100 % with 5α -androsterone, 20 % with dihydrotestosterone, 15.7 % with Δ^4 androstenedione, 3 % with dehydroepiandrosterone and 7.4 % with androsterone. The tracer used was [$1,2,6,7\text{-}^3H$] testosterone (Radiochemical Centre, Amersham, England). The limit of assay sensitivity was 5 pg/ml. The coefficients of

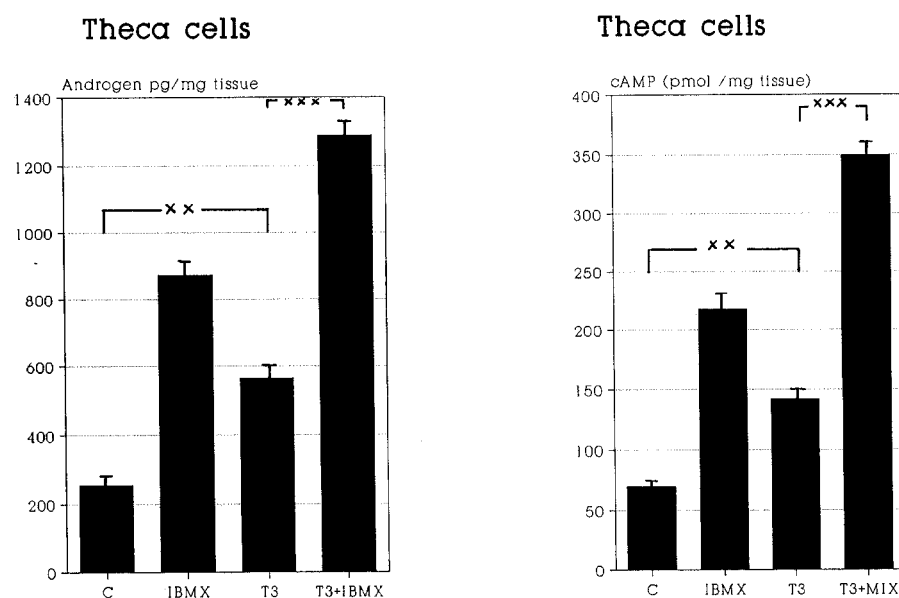


Fig. 1

Androgens secretion and cAMP accumulation in theca cells after the addition of triiodothyronine (10^{-9} M) in the absence or presence of IBMX (0.1mM). Each bar represents the mean \pm SEM of 9 observation from 3 independent experiments. * $P < 0.05$; ** $P < 0.01$, *** $P < 0.001$.

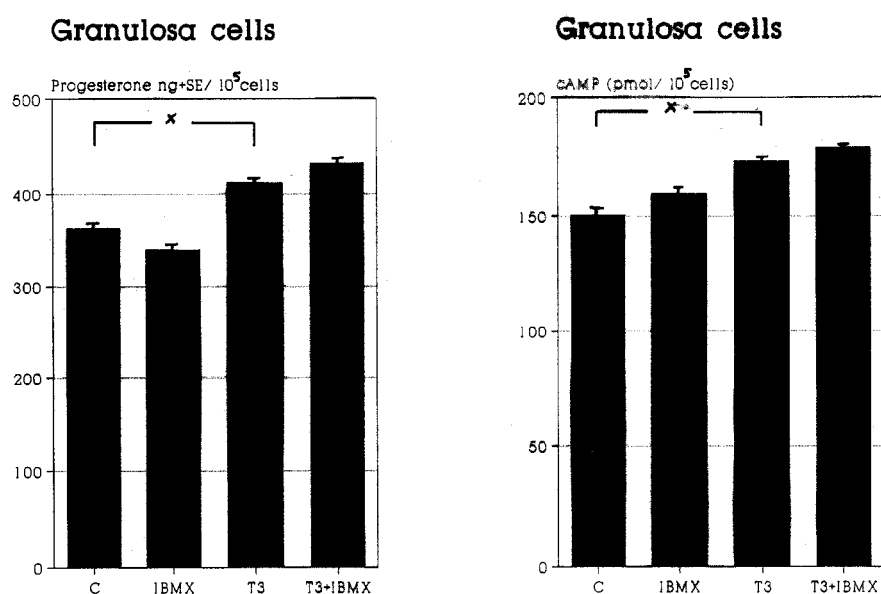


Fig. 2

Progesterone secretion and cAMP accumulation in granulosa cells after the addition of triiodothyronine (10^{-9} M) in the absence or presence of IBMX (0.1mM). Each bar represents the mean \pm SEM of 9 observation from 3 independent experiments. * $P < 0.05$; ** $P < 0.01$, *** $P < 0.001$.

variation within and between assays were 7.5 % and 9.5 % respectively.

Statistical evaluation. All data were expressed as means \pm S.E. derived from at least three different experiments (n=3), each in triplicate, which means at least nine observations. The differences between steroid concentrations in control and treated cultures were evaluated by ANOVA followed by Duncan's new multiple range test.

Results

In theca cell culture, T_3 alone added to the culture media increased androgen secretion (568 pg vs. 256 pg/mg tissue in the control; $P<0.01$) and cAMP accumulation (142 pmol vs. 70.2 pmol/mg tissue in the control; $P<0.01$). Similarly, IBMX added to the culture medium increased the basal and T_3 stimulat-

ed androgen secretion ($P<0.001$) and cAMP accumulation ($P<0.001$) (Fig. 1).

In granulosa cell culture, T_3 alone added to the culture medium only slightly stimulated progesterone secretion (412 ng vs. 363 ng/ 10^5 cells in the control; $P<0.05$) and cAMP accumulation (1735 pmol vs. 1503 pmol/ 10^5 cells in the control; $P<0.05$). The addition of IBMX resulted in a slight but not significant increase of both basal and T_3 stimulated progesterone secretion and cAMP accumulation by Gc (Fig. 2).

In luteal cells culture, the increase of progesterone secretion ($P<0.05$) and cAMP accumulation ($P<0.01$) after the addition of T_3 was observed. IBMX added alone increased progesterone secretion and cAMP accumulation in the same manner as T_3 . Further augmentation of progesterone secretion ($P<0.05$) and cAMP accumulation ($P<0.05$) was observed after the addition of IBMX together with T_3 (Fig. 3).

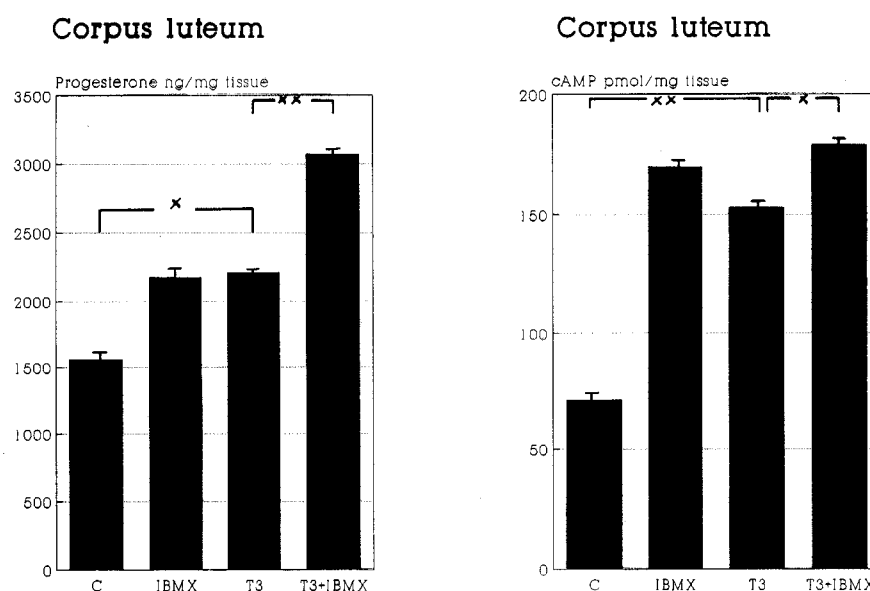


Fig. 3

Effect of addition of triiodothyronine (10^{-9} M), on progesterone secretion and cAMP accumulation by porcine luteal cells cultured in the presence or absence of IBMX. Each value represents mean \pm SEM of 9 observation from 3 independent experiments.

* $P<0.05$; ** $P<0.01$, *** $P<0.001$.

Discussion

The augmenting effect of T_3 on cAMP accumulation could be due to enhanced cAMP synthesis and/or a decrease in cAMP degradation. The contribution of cAMP degradation to the total increase in cAMP accumulation was assessed by the addition of 3-isobutyl-1-methylxanthin (IBMX) to the culture medium, the drug which acts as an inhibitor of PDE enzymes. Ovarian cells in these experiments were cultured in the presence of either T_3 or IBMX alone or of both substances simultaneously. The exposure of Gc to T_3 together with IBMX did not cause any changes in progesterone secretion and cAMP accumulation, whereas without IBMX only slight, but significant effect of T_3 on progesterone secretion and cAMP accumulation was observed. GOLDMAN et al. (1993) did not find any influence of T_3 alone basal progesterone secretion and cAMP accumulation of cultured human granulosa cells harvested from the dominant pre-ovulatory follicles from women undergoing *in vitro* fertilization. They showed that the exposure of cells to T_3 markedly enhanced hCG-induced cAMP accumulation. Modulation of phosphodiesterase activity was suggested as one of mechanism by which thyroid hormones altered cAMP levels, and via cAMP, free fatty acid metabolism in the liver (SHAHEEN et al. 1982). In the presence of IBMX they observed a decrease in the cAMP stimulatory effect elicited by T_3 , suggesting that the thyroid hormone may act in the same way as IBMX by inhibiting phosphodiesterase. There are, however, two major differences between the Goldman's and our study. First, we used porcine granulosa cells and, second, we measured basal but not hormonally stimulated cAMP accumulation. The increase in steroidogenesis of porcine granulosa cells in the presence of T_3 was observed by CHANNING et al. (1976) and MARUO et al. (1987). Surprisingly, there are no data on the mechanism of T_3 action in other follicular compartments, i.e. theca and luteal cells.

In this study, in theca cell culture the increase of T_3 -stimulated androgen secretion and that of cAMP accumulation in the presence of IBMX was observed. The effect of T_3 was lower and the relative effect of IBMX was higher, suggesting a remarkably high activity of PDE rather than the poor responsiveness of adenylate cyclase to T_3 . The shift of T_3 stimulated

cAMP accumulation by theca cells in the presence of IBMX strongly suggests that androgen secretion is mediated by the increasing cAMP synthesis. The additive effect of T_3 and IBMX suggests that both agents can act independently: IBMX by inhibiting cAMP degradation, while T_3 by increasing its synthesis in thecal cells. In luteal cell culture IBMX and T_3 increased androgen secretion and cAMP accumulation in the same manner. The addition of IBMX together with T_3 slightly increased also the secretion of progesterone and accumulation of cAMP. It is conceivable that in luteal cells T_3 acted rather by stimulating cAMP synthesis than by cAMP degradation, since only small augmentation of T_3 induced cAMP accumulation was observed with IBMX.

The influence of T_3 on cyclic nucleotide release by ovarian cells may suggest the involvement of the cAMP in intracellular mechanism taking part in thyroid hormone effects within porcine ovaries.

The data thus suggest that thyroid hormones, by their well known direct effect on the nuclear chromatin (MARUO et al. 1992; WAKIM et al. 1987, 1994a, 1994b) may, like peptide hormones, influence the intracellular cyclic nucleotide synthesis, secretion and metabolism. The biological role of stimulation of cyclic nucleotide production by thyroid hormones may be important. This process could maintain gonadotropin induced steroidogenesis through a positive feedback mechanism. There are some reports that thyroid hormone modulates hCG stimulated progesterone secretion by granulosa cells (GOLDMAN 1993; WAKIM et al. 1995), theca cells (GREGORASZCZUK and SKALKA 1996) and luteal cells (GREGORASZCZUK 1996). CHAN and TAN (1985) suggested the role of thyroid hormone in the action of FSH in the functional differentiation of cultured porcine granulosa cells. It is also possible that, like steroid hormones (SIROTKIN et al. 1995), T_3 could also stimulate gonadotropin receptor proliferation.

Acknowledgements

We wish to thank Professor S.Stoklosowa for invaluable support of this work as well as Dr. M.Slomczynska for help in cAMP assay and Mgr. M.Duda for radioimmunological determinations of steroid hormones. This work was supported by the World Health Organization, SMA Grant, Special

Programme of Research, Development and Research Training in Human Reproduction, and by a grant from the Polish State Research Committee (KBN), 5 POGD 030 14.

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Accepted: March 15, 1998