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EFFECT OF GENISTEIN, TYRPHOSTIN AND HERBIMYCIN ON PROLACTIN-STIMULATED PROGESTERONE PRODUCTION BY PORCINE THECA AND LUTEAL CELLS

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The potential involvement of protein tyrosine kinases (PTK) in the mechanism of prolactin (Prl) action on ovarian cell steroidogenesis has not been elucidated and information about research on this subject is scarce. In this preliminary study pharmacological intervention was used to provide support for a possible involvement of tyrosine kinases in prolactin induction of progesterone secretion by porcine thecal and luteal cells. Material used in this experiment were cultures of porcine follicular theca interna and early corpus luteum cells. The former were isolated from, proestrous preovulatory follicles and the latter were obtained by enzymatic dispersion of luteal tissue. Three of tyrosine kinase inhibitors, genistein, herbimycin and tyrphostin, were applied. They act through different mechanisms, partially blocking Prl-stimulated progesterone secretion. Herbimycin at a dose of 3 μM inhibited Prl-stimulated progesterone secretion beneath the control level in theca and by 70% in luteal cells. Genistein at a dose of 45 μM inhibited Prl-stimulated progesterone secretion beneath the control level in theca and down to the control in luteal cells. On the other hand, tyrphostin at a dose of 100 μM only slightly suppressed Prl-stimulated progesterone secretion by thecal and luteal cells (33% and 40% respectively). This investigation is the first search for evidence of involvement of tyrosine kinases in Prl-stimulated progesterone production by ovarian cells in the pig.

Key words: *prolactin, tyrosine kinase inhibitor, theca cells, luteal cells*

INTRODUCTION

The role of prolactin (Prl) in female reproductive function has long been and still remains the subject of controversy and conflicting evidence. One of the functions of Prl is its role in the regulation of reproductive processes in mammals, and the ovary is one of its target organs. In an earlier study (1, 2),

the direct stimulatory action of Prl on progesterone secretion by theca cells cultured alone, by co-culture with granulosa cells as well as by cultures of luteal cells isolated from early corpora lutea was reported.

Specific binding sites were demonstrated in ovarian tissue (3) which suggested a direct effect of Prl on ovarian steroidogenesis. Prl-receptor (Prl-R) was also detected in rat theca interstitial cells (4) and in theca and luteal cells isolated from the porcine ovary (unpublished). Prl-R belongs to the family of protein kinase receptors together with interleukins, growth hormone and erythropoetin (5—8). Investigation of Prl-R signal transduction in reproductive tissues has developed only recently. Rillema et al., (9) and Rui et al., (10) demonstrated that rapid tyrosine kinase (TK) activation plays a role in the Prl-R signalling pathway and that phosphorylation of proteins on tyrosine residues by protein tyrosine kinase plays an important role in this processes in target cells. According to Ullrich & Schlessinger (11), Marczin *et al.*, (12), Campbell et al., (13) and Levitzki & Gazit, (14), genistein inhibits a wide range of tyrosine kinases, as this isoflavone acts as a competitive inhibitor of the binding of ATP and as a non-competitive inhibitor of the substrate binding to protein tyrosine kinases (11, 16) while herbimycin and tyrphostin have been shown shown to be inhibitors of JAK2 kinases (13). There is a lack of information on the mechanism of Prl action in reproductive glands and organs except of mammary gland and of some cell lines in vitro, this is why any data on this subject seem to have a value. The aim of this paper was observation whether the 3 inhibitors used would suppress Prl-stimulated progesterone secretion, to gain preliminary information which would help to plan further more precise experiments.

MATERIALS AND METHODS

Chemicals

Highly purified ovine prolactin was obtained from Sigma Chemical Co (St. Louis, MO, USA). Tyrphostin, herbimycin A were purchased from GIBCO (USA), genistein from Calbiochem (USA). Medium M199, penicillin, trypsin, calf serum were products of the Laboratory of Sera and Vaccines, Lublin, Poland.

Cell culture preparation

From our earlier experimets (1) it was known that theca cells of large follicles responded to Prl in vitro, therefore proestrous ovaries containing large vascular follicles and corpora lutea albicantia were selected as the source of thecal cells for culture. The separation of granulosa cells from the thecal layer and preparation of theca cell suspension were performed according to our own

technique (17, 18). Newly developed corpora lutea (1—3 days after ovulation) were the source of luteal cells. Dissected corpora lutea were enzymatically dissociated according to the technique of Gregorszczuk (1983).

Thecal and luteal cells were suspended in medium M199 supplemented with 5% of calf serum at a concentration of 3.5×10^5 cells/ml medium.

Cells were grown in multiwell plates (Nunck) in a humidified atmosphere of 5% CO_2 in air.

To asses the effects of genistein (45 μM) or herbimycin A (3 μM) or tyrphostin (100 μM), the compounds were added to the culture medium 30 min before prolactin. Concentration of Prl was used according to Gregoraszczuk et al., (19). Concentration of genistein was established on the basis of the dose response curve performed during preliminary studies (54.4 ng; 34.1 ng; 16.4 ng; 17.3 ng of prosterone/10^5 cells in 15 μM; 45 μM; 75 μM and 100 μM genistein vs. 89.5 ng of progesterone/10^5 cells in Prl-treated cultures). Dose of 45 μM. of genistein was able to inhibit partially, i.e. 50%, of Prl-stimulated progesterone secretion. For tyrphostin and herbinycin doses recomended by Buc et al., (20); Daniel et al., (21); Ruette & Thiemermann, (22) were chosen. After 18 hrs all cultures were terminated and the media were frozen (−20°C) until further steroid analysis. Cell viability of both cell types, measured at the begining and the end of culture was assessed by trypan blue exclusion test and it was 85%.

Progesterone assay

Progesterone assayed by a radioimmunoassay was described elsewhere (18). A highly specific antibody raised in sheep against 11a-hydroxy- progesterone hemisuccinate coupled to bovine serum albumin was used. The cross-reaction with pregnenolone was 2.9%. All other tested steroids showed less than 0.1% cross-reaction. [1, 2, 6, 7-H^3] progesterone (Radiochemical Centre, Amersham, England, sp.act. 80 Ci/mmol) was used as the tracer. The limit of sensitivity of the assay was 50 pg/ml. The coefficients of variation within and between assays were 15% and 2.5%, respectively.

Statistical analysis

All data points are expressed as means ± SEM derived from at least three different replications, each in triplicate, resulting in at least nine observations. Significant differecces between steroid levels in control and treated cells were compared by analysis of variance and using Duncan's new multiple range test.

RESULTS

Effect of tyrosine kinease inhibitors on prolactin stimulated progesterone secretion

Genistein in a dose of 45 μM inhibited by 66% basal progesterone secretion by theca and by 59.9% by luteal cells. In Prl-stimulated cultures genistein

inhibited progesterone secretion beneath the control levels in theca and down to the control level in luteal cells (*Figs. 1, 2*).

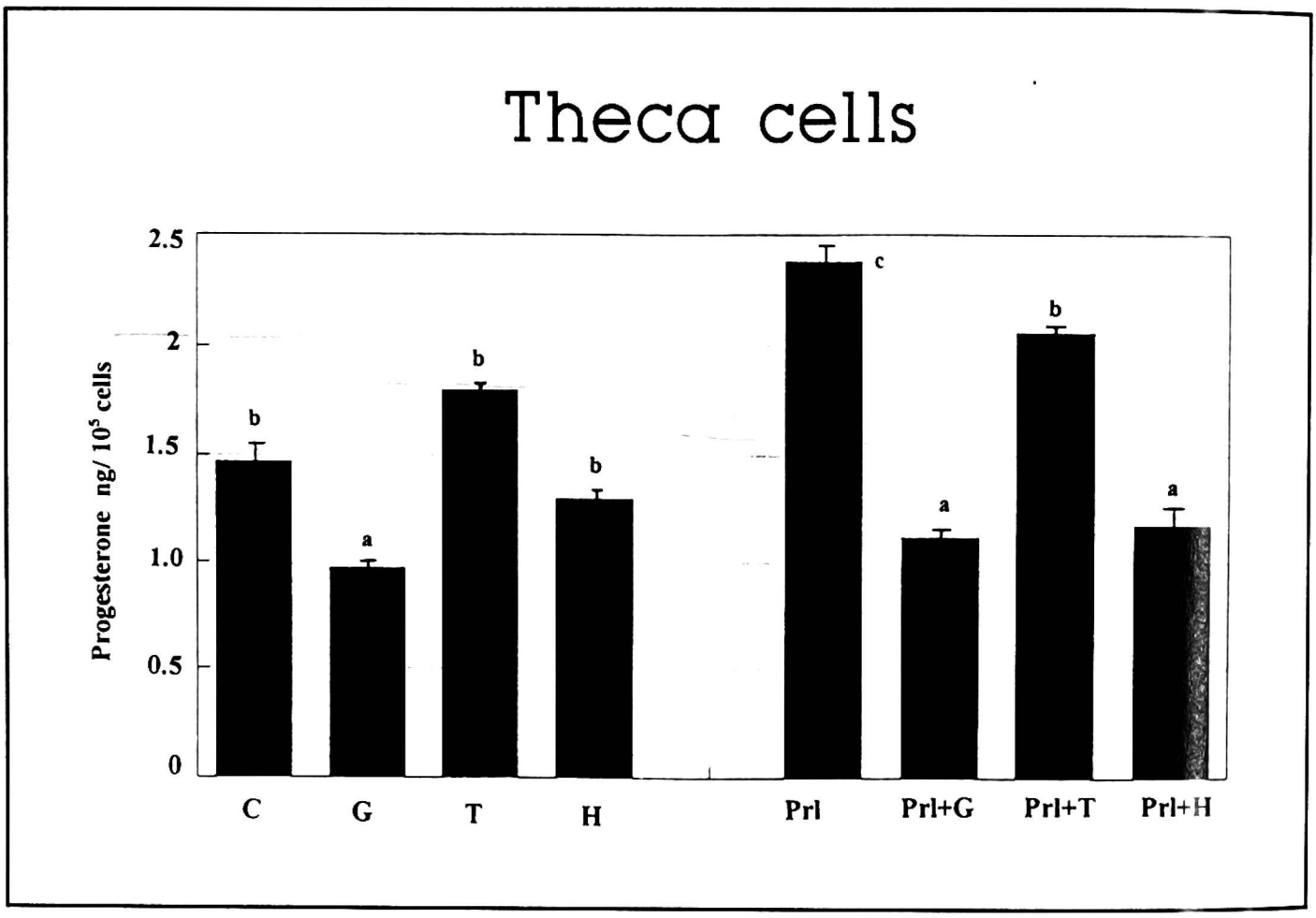

Fig. 1. Effect of tyrosine kinase inhibitors (G-genistein, 45 μM; T-tyrphostin, 100 μM; H-herbimycin, 3 μM) on control (C) and prolactin-stimulated (Prl-100 ng/ml) progesterone secretion by theca cells. The increase of Prl-stimulated progesterone secretion was compared to control level. In the investigation using Prl-stimulated cultures, suppression of progesterone secretion under the inhibitors applied was calculated in relation to the increase of Prl-stimulated progesterone secretion above the control level. Bars with different letters, refer to comparison between C, G, T or H within control or within Prl-stimulated cultures. The data with different superscripts are different ($p < 0.05$) as shown by analysis of variance.

Tyrphostin, on the other hand, in a dose of 100 μM had a small, although not significant stimulatory effect on basal progesterone secretion by theca and no effect on progesterone secretion by luteal cells. Prl-stimulated progesterone secretion was inhibited by 33% in theca and by 40% in luteal cell cultures (*Figs. 1, 2*).

Herbimycin in a dose of 3 μM did not exert a statistically significant effect on basal progesterone secretion in both investigated cell types. However, Prl-stimulated progesterone secretion was inhibited beneath the control level in theca and by 70% in luteal cell cultures (*Fig. 1, 2*).

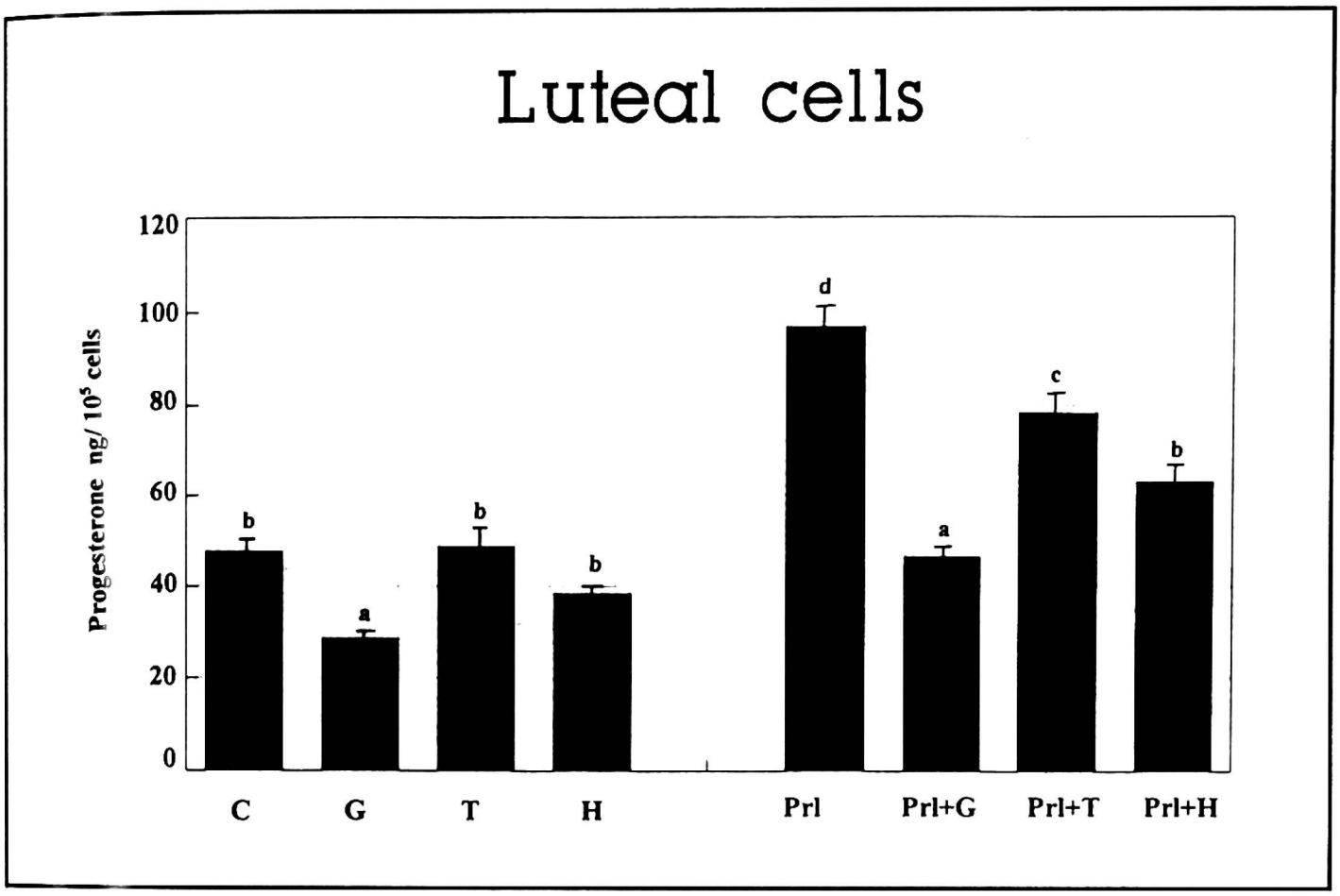

Fig. 2. Effect of tyrosine kinase inhibitors (G-genistein, 45 μM; T-tyrphostin, 100 μM; H-herbimycin, 3 μM) on control (C) and prolactin-stimulated (Prl-100 ng/ml) progesterone secretion by cultured luteal cells. The increase of Prl-stimulated progesterone secretion was compared to control level. In the investigation using Prl-stimulated cultures, suppression of progesterone secretion under the inhibitors applied was calculated in relation to the increase of Prl-stimulated progesterone secretion above the control level. Bars with different letters, refer to comparison between C, G, T or H within control or within Prl-stimulated cultures. The data with different superscripts are different ($p < 0.05$) as shown by analysis of variance.

DISCUSSION

The results of this study suggest that protein tyrosine kinases are required for secretion of progesterone by ovarian cell as affected by Prl. This conclusion is based on the ability of the 3 tyrosine kinase inhibitors (genistein, herbimycin and tyrphostin), suppressing partially Prl-stimulated progesterone secretion.

To assess the biological usefulness of an inhibitor in a system using primary cells, as in the system applied in this experiment, (30 min preincubation), we believe, in accordance with Peterson & Barnes, (23), that a long incubation time should be avoided when investigating rapid and sensitive hormonal activations. This is even more crucial when dealing with effects of PTK inhibitors on basal metabolic processes known as tyrosine kinase independent, which in long term exposure cannot be ruled out. One significant finding emerging from this study is that signal transduction from an expressed prolactin receptor to progesterone production is inhibited not only by

herbimycin, a specific TK inhibitor known to be effective against JAK2 and MAP kinases (13, 20) but also by genistein, which blocks the tyrosine phosphorylation, though is poorly effective against JAK2 kinases.

Herbimycin in a dose of 3 μM inhibited Prl-stimulated progesterone secretion beneath the control level in theca and by 70% in luteal cell cultures. Since prolactin receptor activation is associated with rapid tyrosine phosphorylation of JAK2 (8, 14, 24), it is reasonable to conclude that in ovarian cells herbimycin is exerting its effects through JAK2 inhibition.

Genistein inhibits a wide range of tyrosine kinases, as this isoflavone acts as a competitive inhibitor of the binding of ATP and as a non-competitive inhibitor of the substrate binding to protein tyrosine kinases (11, 15). In the presented data it has been shown that genistein in dose of 45 μM inhibited by 66% basal progesterone secretion by theca and by 59.9% by luteal cells, while in Prl-stimulated cultures genistein inhibited progesterone secretion beneath the control levels in theca and down to the control in luteal cells. The effect of genistein in whole cells has not yet been well documented. The results of this investigation employing intact ovarian cells suggest the involvement of PTK in the Prl-stimulation of progesterone secretion by steroidogenic cells.

Tyrphostin, which is a syntetic PTK inhibitor supressed significantly Prl-stimulated progesterone secretion at 33% by thecal cells and at 40% by luteal cells. This result is in agreement with Negrescu et al., (25) and Levitzki & Gazit, (15) who showed that tyrphostin is only a very weak inhibitor of TK activity *in vitro*. Isoquinolylsuphonamide derivate, known as tyrphostin, exhibits greater selectivity than genistein and acts as a competitive inhibitor of peptide substrate binding (14, 16). The fact that herbimycin, which is known to be effective also against MAP and JAK2 kinases, inhibits Prl-stimulated progesterone secretion to a larger extent than tyrphostin, being most specific inhibitor of TK, suggests JAK2 and MAP kinases participation in Prl action on ovarian cells. The results of our other, yet unpublished, studies allow conclusion that prcine theca and luteal cells express Prl receptors, measured by Schatchard anylsis and by immunocytochemistry. Experiment in search of JAK2 kinases involvement in Prl responsive cells are planned.

The results of this investigation are the first to indicate involvement of TK in Prl-stimulated progesterone production by ovarian cells. Since information about mechanism of Prl action on gonadal cells is scare so these experiments are of pionier character. In further investigation using more specific inhibitors is necessary.

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