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PORCINE THECA CELLS PRODUCE IMMUNOREACTIVE β-ENDORPHIN AND CHANGE STEROIDOGENESIS IN RESPONSE TO OPIOID AGONIST

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In earlier *in vitro* experiments opioids affected steroidogenesis in porcine luteal and granulosa cells. The present studies were undertaken to examine the effects of FK 33–824 (opioid agonist) alone or in combination with LH, PRL or naloxone (NAL, opioid antagonist) on steroidogenesis in cultured porcine theca cells. Moreover, we have tested β-endorphin-like immunoreactivity (β-END-LI) concentrations in culture media under control conditions and following treatments of theca cells with LH, PRL, progesterone (P_4), oestradiol (E_2) or testosterone (T). FK 33-824 and NAL significantly increased P_4 release by theca cells and inhibited stimulatory effect of LH on this steroid output. PRL-induced P_4 secretion from the cells was blunted only by FK 33-824. Secretion of androstenedione (A_4) and T was essentially elevated in the presence of FK 33-824 and this potentiation of both androgen release was completely abolished by PRL. NAL blocked stimulatory effect of the opioid agonist only in case of T. Secretion of oestradiol and oestrone was completely free from the influence of both the opioid agonist and antagonist. Pig theca cells were able to produce β-END-LI but none of tested hormones (LH, PRL, P_4, E_2 and T alone or in combination) significantly affected this production. In conclusion, these data indicate that porcine theca cells may produce β-END-LI and change their steroidogenesis in response to opioid peptides.

Key words: Opioid peptides, β-endorphin, porcine theca cells, steroid secretion

It is well known that opioid peptides regulate the hypothalamo-pituitary-gonadal axis, mainly through the modulation of GnRH and LH secretion (Brooks et al., 1986; Barb et al., 1991; Okrasa et al., 1995). In addition, there is a growing body of evidence, which indicates a participation of opioid peptides in the regulation of reproductive function through a direct local action within reproductive tissues. The genes for the three known opioid peptide precursors – proopiomelanocortin, proenkephalin, and prodynorphin – are all expressed in the female reproductive system (Melner et al., 1986; Jin et al., 1988; Sanders et al., 1990;

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Douglass et al., 1987). Opioid peptides derived from each of these precursors have been detected in various tissues and fluids of the reproductive tract (Lim et al., 1983; Ehrenreich et al., 1985; Petraglia et al., 1985; Lolait et al., 1986; Li et al., 1991; Slomczynska et al., 1997), and changes in the concentration of some of these peptides in gonads of cyclic females have also been reported (Petraglia et al., 1985; Petraglia et al., 1986; Lolait et al., 1985; Lovegren et al., 1991; Przała et al., 2001; Kaminski et al., 2000). Reports concerning the influence of opioids on steroidogenesis in human (Facchinetti et al., 1986) and porcine (Gregoraszczuk and Slomczynska, 1998; Kaminski et al., 2000) granulosa cells, as well as in rat (Kato et al., 1993), bovine (Varsano et al., 1990) and porcine (Kaminski et al., 1999) luteal cells suggest that opioid peptides exert autocrine and/or paracrine effects in the ovary. However, to our knowledge, there is no information on the influence of opioids on steroidogenesis in theca interna cells. A study was thus designed to examine:

(1) the effect of FK 33-824 (opioid agonist), which interacts with μ and δ opioid receptors (Roemer et al., 1977; Wood et al., 1981), and naloxone (NAL, blocker of opioid receptors) on secretion of progesterone, androstenedione, testosterone, oestradiol-17β, and oestrone by theca interna cells from large porcine follicles,

(2) the ability of porcine theca cells from large follicles to produce immunoreactive β-endorphin and the possible influence of LH, PRL, progesterone, oestradiol, and testosterone on immunoreactive β-endorphin release by these cells.

Materials and methods

Materials

Naloxone (opioid antagonist), FK 33-824 (opioid agonist), insulin, hydrocortisone, transferrin, progesterone, androstenedione, testosterone, oestradiol-17β, oestrone, bacitracin and 24-well culture plates were purchased from Sigma (St. Louis, MO, USA). Prolactin (30 IU/mg) was isolated from porcine pituitaries and kindly provided by Prof. Kazimierz Kochman (The Kielanowski Institute of Animal Physiology and Nutrition, Jablonna near Warsaw, Poland). Porcine LH was kindly supplied by the National Hormone and Pituitary Agency, University of Maryland, USA. Labelled hormones: (1,2,6,7-^{3}H) progesterone, (1,2,6,7 (N)-^{3}H) androst-4-ene-3,17 dione, (1,2,6,7-^{3}H) testosterone, (2,4,6,7-^{3}H) oestrone, and (2,4,6,7-^{3}H) oestradiol were from Amersham, UK. Porcine β-endorphin and antisera against β-endorphin were purchased from Peninsula Laboratories Inc. (Belmont, CA, USA). Eagle's medium and trypsin were products of the Laboratory of Sera and Vaccines (Lublin, Poland), BSA fraction V was from the Laboratory of Sera and Vaccines (Kraków, Poland), antibiotics were from Polfa (Poland) and trypan blue from Chemapol, Czech Republic.

Isolation and incubation of theca cells

Ovaries, harvested from locally slaughtered crossbred pigs, were placed in cold buffered physiological saline (PBS) supplemented with antibiotics and immediately transported to the laboratory. The stage of the oestrous cycle was determined according to morphological criteria (Akins and Morrissette, 1968). Theca cells were isolated from large follicles (diameter > 6 mm) without signs of atresia using a slight modification of the technique described by Stoklosowa et al. (1978). Follicles were classified as non-atretic if they had extensive and very fine vascularization, a regular granulosa cell layer and no free-floating particles in the follicular fluid. Theca interna layer was scraped off from granulosa cells and enzymatically dispersed in 0.25% trypsin solution. Dispersed cells were filtered through nylon mesh (40 μm in diameter), washed three times and resuspended in the incubation medium: Eagle's medium containing BSA fraction V (5%), hydrocortisone (40 ng/ml), insulin (2 μg/ml), transferrin (5 μg/ml), and antibiotics. Additionally, in the experiments examining the effects of LH, PRL and steroids on β-END-LI release by theca cells, bacitracin (28 μg/ml) was added to the incubation media. Medium composition was chosen according to our earlier experiences and to results reported by Wiesak et al. (1994) and Baranao and Hammond (1985). The cells were counted using a haemocytometer and their viability (99%) was determined by 0.4% trypan blue dye exclusion. Theca cells (10^5 viable cells/well in the experiment examining steroid secretion and 10^6 viable cells/well in the experiment examining β-endorphin release) were cultured in 24-well culture plates for 24 h in serum-free conditions in a humidified incubator (37 °C) gassed with 95% air and 5% CO_2. Incubations were carried out in duplicate, and all experiments were repeated eight to twelve times. Following incubation in the presence or absence of stimulants, the media were harvested, centrifuged (800 × *g* for 10 min) and the supernatants were collected and stored at –20 °C until RIA analyses. None of the treatments affected the viability of the cells determined by trypan blue dye exclusion.

Experiment 1: Effects of FK 33-824 and NAL alone and in combination with LH and PRL on steroid secretion by theca cells

In this experiment the cultured cells were divided into nine groups treated with FK 33-824 alone (10^{-9} M), NAL alone (10^{-5} M), FK 33-824 with NAL, LH alone (100 ng/ml), LH with FK 33-824 or NAL, PRL alone (100 ng/ml), and PRL with FK 33-824 or NAL. Control group of the cells was incubated without mentioned above agents. The dose of each factor was established in our preliminary dose-response experiments or based on data published by Facchinetti et al. (1986).

Experiment 2: Effects of LH, PRL and steroids on β-END-LI secretion by theca cells

In this experiment LH, PRL (both at the concentrations 1, 10, 100 ng/ml), progesterone, oestradiol and testosterone (all steroids at the concentrations 10^{-9}, 10^{-7}, 10^{-5} M) alone or LH (100 ng/ml) in combination with P_4, E_2 or T (10^{-9}, 10^{-7}, 10^{-5} M) were added to the incubation media. The medium of the control group did not contain LH, PRL and the above-mentioned steroids.

Radioimmunoassays of steroid hormones

Progesterone and oestradiol-17β concentrations were determined according to the method of Hotchkiss et al. (1971) modified by Kotwica (1987), those of A_4 and E_1 as described by Dziadkowiec et al. (1982) and those of T according to Kotwica and Williams (1982).

Cross-reactivities of the antiserum against P_4 have been published previously (Dziadkowiec et al., 1982). The specificity of the antibodies against A_4, T, E_2 and E_1 has been reported by Szafranska et al. (in press).

Validity of the assays was confirmed by parallelism between the standard curves and a series of dilutions of randomly chosen samples.

Intra- and inter-assay coefficients of variation of the P_4, A_4, T, E_2 and E_1 assays were 5.52%, 3.20%, 2.19%, 4.14% and 3.14% and 15.09%, 8.68%, 5.15%, 5.87% and 9.21%, respectively. The sensitivities of the assays for P_4, A_4, T, E_2 and E_1 were 22 pg/ml, 4.5 pg/ml, 3.9 pg/ml, 4.2 pg/ml and 2.4 pg/ml, respectively.

Radioimmunoassay of β-END-LI

β-Endorphin-like immunoreactivity (β-END-LI) in media was established by the RIA procedure previously described by Okrasa et al. (1995) and Przała et al. (2001). The rabbit antiserum against β-endorphin exhibited equimolar cross-reactivity (100%) with β-endorphin and β-lipotropin but did not exhibit any cross-reactivity with α-endorphin, γ-endorphin, met-enkephalin, ACTH and α-MSH. Porcine β-endorphin was used for iodination and standards.

Because of small amounts of β-END-LI released from theca cells, incubation media were lyophilised before the assay. Samples were then reconstituted with 200 µl of assay buffer. Serial dilutions of chosen samples showed parallelism with the standard curve.

The sensitivity of the assay and the intra- and inter-assay coefficients of variation were 20 pg/ml (at 92% binding), 8.52% and 16.21%, respectively.

Statistical analysis

All data for experiment 1 (n = 12) and experiment 2 (n = 8) were analysed by one-way analysis of variance and Duncan's test and are presented as mean ± SEM. $P < 0.05$ was considered to be statistically significant.

Results

Experiment 1: Effects of FK 33-824 and NAL alone and in combination with LH and PRL on steroid secretion by theca cells

The addition of FK 33-824 was followed by a significant increase in P_4 release by theca cells. Similar response of the cells was also observed after NAL treatment. However, simultaneous addition of FK 33-824 and NAL was without effect on the steroid output compared with the control group. Moreover, FK 33-824 inhibited the stimulatory effect of LH and PRL on P_4 secretion. In case of NAL, such an effect was noted only in the presence of PRL (Fig. 1).

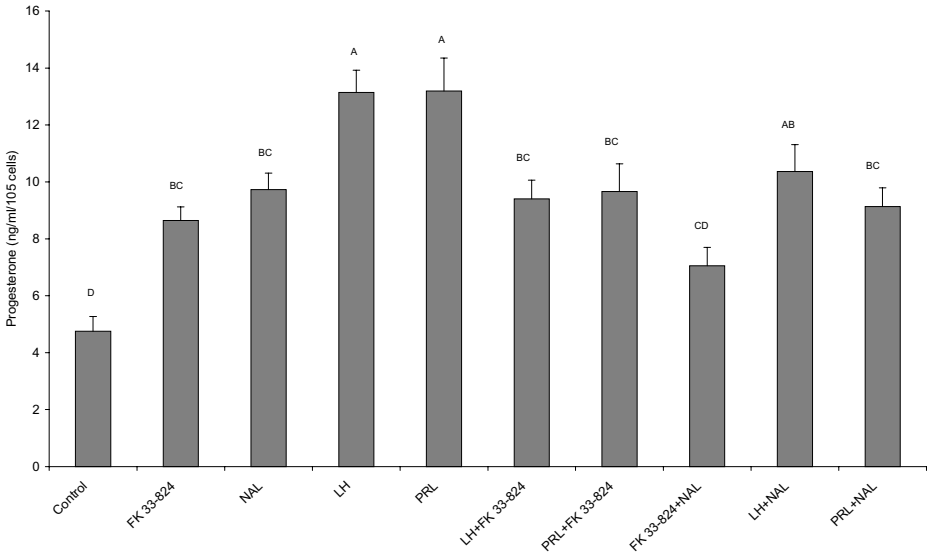

Fig. 1. Effect of FK 33-824 alone or in combination with naloxone (NAL), LH and PRL on progesterone secretion by cultured theca cells from large follicles. Results are means ± SEM (n = 12). Bars with different superscripts are significantly different ($p < 0.05$)

Androstenedione secretion was essentially elevated under the influence of FK 33-824. This stimulatory effect of the opioid agonist was reduced in the presence of PRL. NAL alone did not affect A_4 release. Nevertheless, NAL significantly depressed stimulatory effect of LH (Fig. 2).

The release of the second examined androgen, testosterone, was also potentiated by FK 33-824 and this potentiation was completely abolished by NAL and PRL (Fig. 3).

Secretion of E_2 and E_1 was entirely independent of both opioid agonist and antagonist (data not shown).

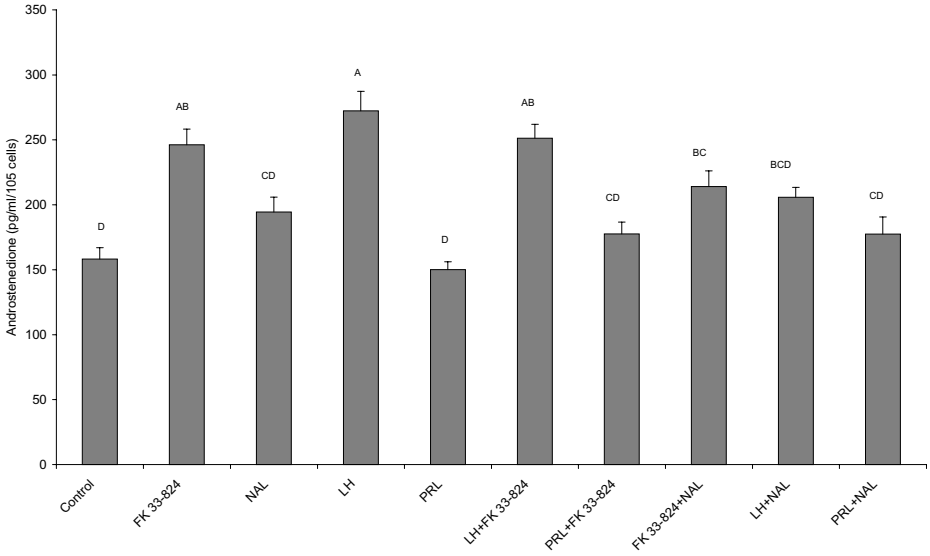

Fig. 2. Effect of FK 33-824 alone or in combination with naloxone (NAL), LH and PRL on androstenedione secretion by cultured theca cells from large follicles. Results are means ± SEM (n = 12). Bars with different superscripts are significantly different ($p < 0.05$)

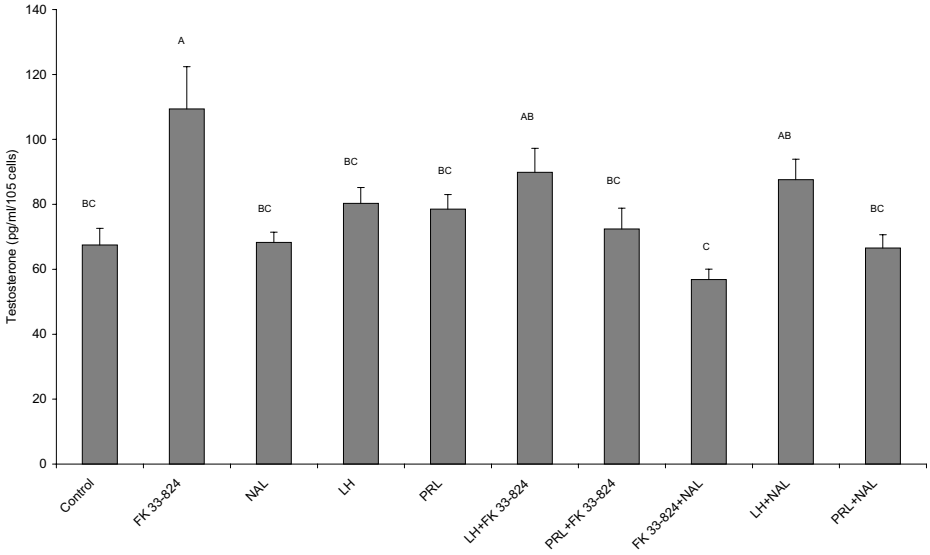

Fig. 3. Effect of FK 33-824 alone or in combination with naloxone (NAL), LH and PRL on testosterone secretion by cultured theca cells from large follicles. Results are means ± SEM (n = 12). Bars with different superscripts are significantly different ($p < 0.05$)

Experiment 2: Effects of LH, PRL and steroids on β-END-LI secretion by theca cells

Porcine theca cells exhibited β-END-LI secretion *in vitro* under basal (i.e. control) conditions. Treatment with LH, PRL, P_4, T and E_2 alone (Table 1) and in combination (Table 2) did not significantly affect secretion of β-END-LI by these cells. However, a tendency for increase ($p = 0.094$) has occurred in the presence of the highest dose (100 ng/ml) of LH (Table 1), which was abolished by co-treatment with steroid hormones (Table 2). Moreover, a tendency for decrease ($p = 0.056$) by PRL at the concentration 100 ng/ml has been observed (Table 1).

Table 1

Secretions of β-END-LI (means ± SEM, n = 8) by porcine theca cells in the presence of protein (LH, PRL) and steroid hormones (P_4, E_2, T) alone

Treatments	β-END-LI concentrations in media (pg/ml/10^6 cells)			
	Control	Doses of tested hormones		
		1 ng/ml	10 ng/ml	100 ng/ml
–	114.20 ± 8.09			
LH		120.61 ± 7.28	132.52 ± 12.14	132.41 ± 11.42
PRL		96.91 ± 9.73	96.15 ± 11.54	90.89 ± 17.31
		10^{-5} M	10^{-7} M	10^{-9} M
P_4		101.14 ± 12.49	109.22 ± 17.69	100.91 ± 15.59
E_2		103.28 ± 19.71	105.17 ± 13.58	106.48 ± 19.38
T		101.48 ± 19.58	94.52 ± 17.46	123.92 ± 16.51

Table 2

Secretions of β-END-LI (means ± SEM, n = 8) by porcine theca cells in the presence of LH (100 ng/ml) and different doses of steroid hormones (P_4, E_2, T)

Treatments	β-END-LI concentrations in media (pg/ml/10^6 cells)			
	Control	Doses of tested steroids		
		10^{-5} M	10^{-7} M	10^{-9} M
None	114.20 ± 8.09	–	–	–
LH	132.41 ± 11.42	–	–	–
LH + P_4	–	104.28 ± 19.25	101.33 ± 17.31	100.28 ± 15.56
LH + E_2	–	116.44 ± 22.28	123.37 ± 19.36	111.32 ± 24.15
LH + T	–	116.98 ± 27.11	107.40 ± 18.52	111.92 ± 25.24

Discussion

In this study we examined the influence of the opioid agonist, FK 33-824, added alone or together with NAL, LH or PRL on steroid hormone release by porcine theca cells and, on the other hand, the effects of LH, PRL and ovarian steroid hormones on β-END-LI secretion by the cells. FK 33-824 stimulated basal secretion of P_4, A_4 and T by untreated theca cells and affected the steroid output by these cells primed with LH or PRL. Only oestrogen release was not altered by treatment with the opioid agonist. The interaction of FK 33-824 with LH or PRL resulted in different changes in P_4, A_4 and T secretion by theca cells. In case of P_4 release, FK 33-824 attenuated stimulatory effect of LH and PRL. On the other hand, the stimulatory effect of the opioid agonist on A_4 and T output was blocked in the presence of PRL. These data are very similar to our previously published observations pertaining to porcine granulosa cells, which have shown that FSH- or PRL-induced P_4 production was blocked by FK 33-824 and the opioid agonist-stimulated androgen secretion was inhibited by PRL (Kaminski et al., 2000). Interactions between opioids and gonadotropins were also noted in earlier studies performed by Kaminski et al. (1999) on porcine small luteal cells, by Gregoraszczuk and Slomczynska (1998) on pig granulosa cells, by Facchinetti et al. (1986) on human granulosa cells, by Kato et al. (1993) on rat luteal cells and by Varsano et al. (1990) on bovine luteal cells. Collectively, our findings coupled with above-mentioned studies strongly suggest a participation of opioids in paracrine/autocrine regulation of ovarian cells.

Interaction of opioid peptides with LH and PRL, pointed out in the present study, suggests the existence of cross-talk between signalling systems induced by opioids and LH or PRL. Our recent studies have suggested that adenyl cyclase and phosphoinositide-specific phospholipase C are involved in intracellular mechanisms of opioid action on porcine luteal (Kaminski et al., 1999) and theca cells (Kaminski et al., unpublished information). It is also well known that activities of these enzymes are affected by LH (Westfall et al., 1994; Davis et al., 1996) and/or PRL (Boyle-Feysot et al., 1998) following binding to their receptors. Thus, interaction between opioids and pituitary hormones seems to be possible at the level of intracellular signalling systems.

In the present study the effects of FK 33-824 and naloxone on P_4 release from theca cells were surprisingly parallel (stimulatory) just as in experiments on pig granulosa cells (Kaminski et al., 2000). One possible explanation for this unexpected similar action of the opioid agonist and antagonist may result from the different binding affinities of FK 33-824 and NAL to opioid receptors. While FK 33-824 binds to μ receptors and, to a lesser degree, to δ receptors, NAL at the concentration used blocks all main types (μ, δ, κ) of opioid receptors. Previous studies on granulosa and luteal cells confirmed the presence of these types of opioid receptors in the porcine ovary (Hamada et al., 1995; Slomczynska et al.,

1997). If κ receptors exist on porcine theca cells, theoretically they might be responsible for non-antagonistic action of NAL in comparison with the opioid agonist effect on P_4 secretion by these cells. It is also worth noting that the influences of NAL on progesterone (stimulation) and on both androgen (no effect) secretion were not the same. Thus, this observation implies that opioids have differentiated effects on steroidogenic enzymes; P_{450scc} and 3βHSD connected with P_4 production and P_{450c17} responsible for androgen synthesis.

In the present experiment, besides the aforementioned susceptibility to the modulatory influence of opioid compounds, porcine theca cells have the potential to secrete β-END-LI, comparable to that of pig granulosa cells (Kaminski et al., 2000). However, none of treatments used (LH, PRL, P_4, T, E_2) significantly affected β-END-LI secretion by porcine theca cells. LH and PRL brought about some changes (approx. 20%) in β-END-LI secretion; increase and decrease, respectively. The stimulatory effect of LH was abrogated by addition of steroids. In a study on pig granulosa cells FSH many-fold increased β-END-LI output in a dose-dependent manner. We also observed that stimulatory effect of FSH was inhibited by P_4 (Kaminski et al., 2000). In other studies on pig luteal cells β-END-LI output was affected by hCG, TNFα (Przała et al., 1999), PRL, oxytocin and progesterone (Przała et al., 2001). Thus, in contrary to granulosa and luteal cells, β-END-LI secretion by porcine theca cells seems to be autonomous or dependent on other regulatory factors not considered in the present studies.

In conclusion, our data indicate that opioid peptides can have a meaningful effect on steroid hormone secretion by porcine theca cells, which also produce β-endorphin representing this group of peptides. Generally, the present results support the possibility that opioid peptides may act as ovarian autocrine/paracrine factors regulating follicular steroidogenesis.

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