LARGE LUTEAL CELLS ARE THE SOURCE OF IMMUNOREACTIVE β-ENDORPHINE IN THE PIG: EFFECTS OF HCG AND TNFA ON ITS SECRETION BY LUTEAL CELLS *IN VITRO*.

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Objective. 1. To compare the release of β-endorphin-like immunoreactivity (β-END-LI) by large and small luteal cells of the pig; 2. to test the effects of human chorionic gonadotropin (hCG) either alone or combined with the cytokine TNFα on β-END-LI secretion by these cells.

Methods. Isolated large and small luteal cells on days 8-10 of the cycle (n=7) were incubated in M199 supplemented with hydrocortisone (40 ng/ml), transferrin (5 μg/ml), insulin (2 μg/ml), gentamicin (50 μg/ml), nystatin (240 U/ml), porcine low-density lipoproteins (LDL; 100 μg/ml), 1 % BSA and 2 x 10^{-5} M bacitracin, for 12 h at 37 °C and under the atmosphere of 95 % air and 5 % CO_2. The cells were treated either with hCG (100 ng/ml) or TNFα (0.1, 1 and 3 nM) alone or with both agents together. β-END-LI concentration in incubation media was measured by RIA.

Results. β-END-LI secretion by large luteal cells was 15-fold greater than by small cells (666.38 ± 24.59 pg/ml/10^6 cells *vs.* 44.60 ± 2.53 pg/ml/10^6 cells). hCG enhanced β-END-LI secretion by both large and small luteal cells. TNFα alone had no effect on β-END-LI release by large and small luteal cells, but it abolished the stimulatory effect of hCG on β-END-LI secretion by large luteal cells.

Conclusion. The results indicate that large luteal cells are a major source of β-endorphin in the porcine corpus luteum, where its release may be affected by gonadotropins (possibly LH) and TNFα.

Key Words: Luteal cells – hCG – TNFα – β-endorphin – Gilts

Endogenous opioid peptides (EOPs) were first identified in the central nervous system (HUGHES et al. 1975) and then were also found in various peripheral tissues, including ovarian structures, where they are implicated in local regulatory interactions (BARDIN et al. 1987; MARGIORIS 1993). Immunoreactive β-endorphin, one of the most extensively studied EOPs, has been detected within the corpora lutea of rodents (SHAHA et al. 1984; LOLAIT et al. 1985), sheep (LIM et al. 1983), cows (EHRENREICH et al. 1985) and pigs (PRZALA et al. 1998).

Gonadotropic influence seems to participate in the control of ovarian production and/or secretion of opioid peptides. It has been confirmed that hCG, PMSG and FSH may augment the release of β-endorphin from rat (LOVEGREN et al. 1991; KATO et al. 1993) and porcine ovaries (KAMINSKI et al., unpublished).

Tumor necrosis factor-α (TNFα) is a cytokine, which has also been identified within the ovary (BAGAVANDOSS et al. 1988, 1990; ROBY and TERRANOVA 1989; ROBY et al. 1990; KONDO et al. 1995). HEHNKE-VAGNONI et al. (1995) localized TNFα in endothelial cells of luteal tissue collected from cyclic and pregnant pigs. KNOKE et al. (1993) also identified TNFα mRNA in porcine large luteal cells with PCR method. Moreover, specific receptors for TNFα have been identified in membranes isolated from porcine corpora lutea (RICHARDS and ALMOND 1994b), as well as in undifferentiated granulosa cells isolated from follicles of immature pigs (VELDHUIS et al. 1991). The results of these studies suggest that TNFα is involved in modulating ovarian function, acting – in addition to an endocrine manner – even in autocrine and/or paracrine way (KLASING and JOHNSTONE 1991). Thus,

TNFα may belong to a group of local factors which affect luteal β-endorphin production and/or release.

The present studies were undertaken: 1. to investigate the source of β-endorphin in the porcine corpus luteum, 2. to test the effects of hCG and TNFα on β-endorphin secretion by porcine small and large luteal cells *in vitro*.

Materials and Methods

Tissues. Ovaries were dissected in the local slaughterhouse from mature crossbred gilts on days 8-10 of the estrous cycle and transported to the laboratory in cold PBS with penicillin (50 IU/ml), streptomycin (50 μg/ml) and gentamicin (50μg/ml). The stage of the estrous cycle was established according to the tables published by Akins and Morrissette (1968).

Luteal cell dispersion. Corpora lutea were dissected from the ovaries, weighed, minced into small fragments (1-2 mm) and dispersed by using 0.125 % trypsin solution in F-12 medium supplemented with gentamicin (50 μg/ml) and nystatin (240 U/ml). Luteal cells were obtained by sequential dissociation of the luteal tissue (4 to 6 times, 10 min each) at 37 °C and centrifuged (800xg for 10 min). The cells were rinsed three times with fresh medium, filtered through a nylon mesh (75 μm) to remove undigested fragments of tissue and counted by using a haemocytometer. Cell viability was determined by trypan blue dye exclusion and it was found to be greater than 98 %.

Cell separation. Concentrated cells (5×10^7) were resuspended in 50 ml of 1 % Ficoll 400 and then transferred into a previously prepared linear density gradient. Initial densities of Ficoll 400 used to prepare the gradient were 2 and 4 %. The distribution of cells was carried out using the Celsep system (DuPont, USA) for two hours at room temperature. Fractions (20 ml) were collected and initially washed three times with M199 containing 1 g BSA/100 ml. Fractions 2-5 contained large luteal cells (LLCs; >30 μm in diameter) and fractions 17-28, small luteal cells (SLCs; 10-20 μm). The viability of SLCs and LLCs after separation was 100 % and 96.87 ± 2.23%, respectively. Contamination of LLC fractions by small cells was 15.78 ± 4.58 % and the fractions of SLCs were completely free of contamination by large cells.

Effects of hCG and TNFα on β-END-LI secretion. Small luteal cells (10^6 cells/well) and LLCs (10^5 cells/well) were incubated in 1 ml of incubation medium in 24-well plastic plates for 12 h, under a humidified atmosphere of 5 % CO_2 and 95 % air, at 37 °C. The incubation medium (M199) was supplemented with hydrocortisone (40 ng/ml), transferrin (5 μg/ml), insulin (2 μg/ml), gentamicin (50 μg/ml), nystatin (240 U/ml), porcine LDL (100 μg/ml), 1% BSA and bacitracin (2×10^{-5}M). Small and large luteal cells were treated with hCG (100 ng/ml) and TNFα (0.1, 1 and 3 nM), alone and with both agents together. Doses of TNFα were chosen according to Pitzel et al. (1993). hCG was kindly provided by the National Hormone and Pituitary Agency, NIH (University of Maryland, School of Medicine, USA) and TNFα was purchased from Sigma (St. Louis, MO, USA). All incubations were performed in duplicate. Following incubation, media were harvested (800 x g/10 min) and the supernatants were collected and stored at -20 °C until RIA analysis.

β-endorphin RIA. β-endorphin-like immunoreactivity (β-END-LI) was estimated by the RIA method previously described by Ostrowska et al. (1990) and modified by Okrasa et al. (1995), in which a second antibody procedure is used to separate free from bound labeled β-endorphin. Rabbit antiserum against β-endorphin, which exhibited equimolar cross-reactivity (100 %) with β-endorphin and β-lipotropin, was purchased from Peninsula Laboratories Inc. (Belmont, California, USA). The antiserum against rabbit gamma-globulin was produced in our Department. Porcine β-endorphin was used for iodination and standards (Peninsula Laboratories Inc.). Incubation media were lyophilized before assay to concentrate β-END-LI. Samples were subsequently reconstituted with 200 μl of assay buffer. The sensitivity of the assay and the intra-assay and inter-assay coefficients of variation were 20 pg/ml (at 92 % binding), 8.52 % and 16.21 %, respectively.

Statistical evaluation. The amounts of β-END-LI secreted by LLCs were recalculated for 10^6 cells to obtain comparable values for both luteal cell types studied. All data points were expressed as mean±S.E. of at least seven replicates. Comparisons of mean values were carried out by ANOVA followed by the LSD test. Significant differences were assumed for $P<0.05$ and highly significant differences for $P<0.01$.

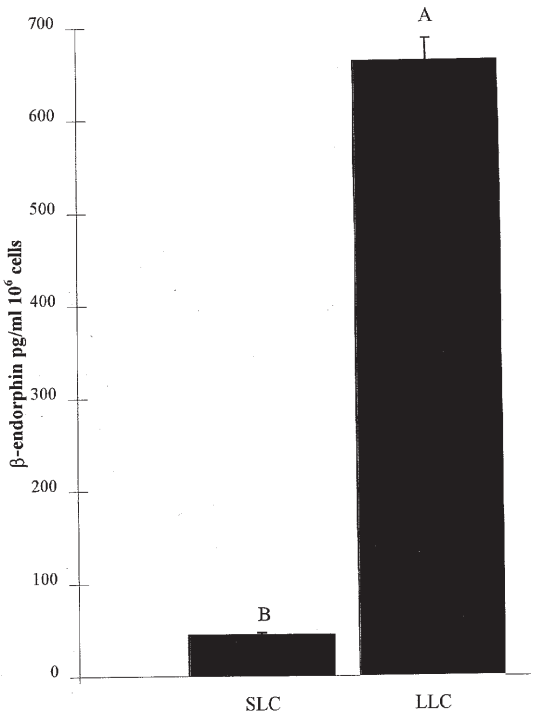

Fig. 1 Basal β-END-LI secretion by porcine SLC and LLC during culturing for 12 h. Results are means±S.E. of seven replications. Bars with different superscripts represent significantly different (P<0.001) values.

Results

As shown in Fig. 1, β-END-LI secretion by LLCs (10^6 cells) was 666.38 ± 24.56 pg/ml medium, this being about 15-fold higher (P<0.001) than the amount (44.60 ± 2.53 pg/ml) produced by the same number of SLCs.

Treatment with hCG increased (P<0.05) β-END-LI release *in vitro* from SLCs by approximately 21 % (Fig. 2). TNFα alone, irrespective of the dose used, had no effect on β-END-LI secretion by SLCs. In the presence of both hCG and TNFα, SLCs secreted intermediate amounts of β-END-LI which did not significantly differ from those observed in response to separate treatment of the cells with these substances (Fig. 2).

Large luteal cells, similarly to SLCs, increased β-END-LI secretion in response to hCG by 27 % (Fig. 3). Low doses of TNFα alone (0.1 and 1 nM) did not change β-END-LI release from LLCs, but added in combination with hCG they abolished (P<0.05) the stimulatory effect of this gonadotropin on β-END-LI secretion. However, in the presence of a higher TNFα concentration (3 nM) LLCs tended to increase secretion of β-END-LI (740.19 ± 48.65 pg/ml *vs.* 662.38 ± 24.56 pg/ml for the control), but this change was not statistically significant. The higher dose of TNFα (3 nM) used concomitantly with hCG did not significantly suppress hCG-induced β-END-LI secretion by LLCs, as observed in the case of lower doses of this cytokine.

Discussion

The present results demonstrate that porcine luteal cells synthesize and release β-END-LI and also indicate that LLCs are main source of this peptide in the porcine corpus luteum. They secreted 15 times more β-END-LI than SLCs and about 6 times more than mixed luteal cells cultured under comparable conditions (Przala et al. 1998). In fact, the real amounts of β-END-LI produced by LLCs might be a little higher than that stated above, since the LLC fractions used were contaminated with approximately 15 % of small luteal cells. Thus, in addition to our previous data concerning the luteal content of β-END-LI and its secretion by luteal cells *in vitro*, the present results more precisely define the local origin of luteal β-endorphin. The porcine corpus luteum not only produces β-endorphin but also possesses a single class of ^{3}H-naloxone binding sites [K_d=28.5 x 10^{-9} mol/l] highly specific for this peptide, as proved in displacement reaction (Hamada et al. 1995). However, it cannot be entirely excluded that even some other opioids, besides of β-endorphin, play some role in the function of the porcine corpus luteum. For example, Slomczynska et al. (1997), using immunocytochemical methods, found kappa-opioid receptors in developing porcine follicles and the prodynorphin derived peptide α-neoendorphin in follicular fluid during the estrous cycle.

β-Endorphin production in the porcine corpus luteum appears to be at least under partial control by gonadotropins. In the present studies hCG stimulated β-END-LI release from both small and large luteal cells, by 21 % and 27 %, respectively. Rat luteal cells have also shown increased β-endorphin release in response to treatment with hCG (Kato et al. 1993). The capacity of LH to enhance POMC gene expres-

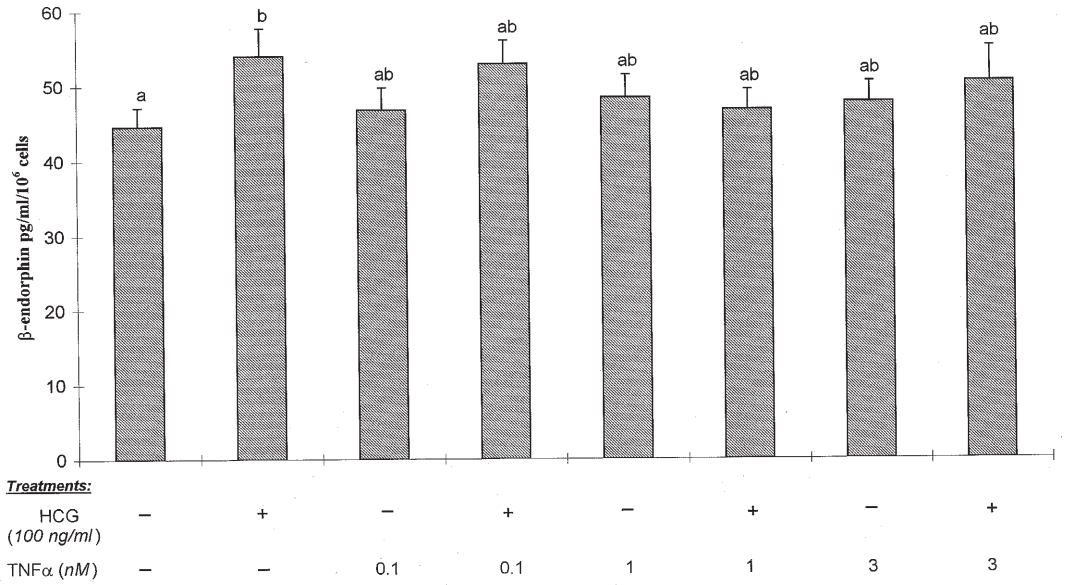

Fig. 2 Effect of hCG on β-END-LI secretion by porcine SLC in the absence or presence of tumor necrosis factor-α (TNFα). Results are means±S.E. of seven replications. Bars with different superscripts represent significantly different (P<0.05) values.

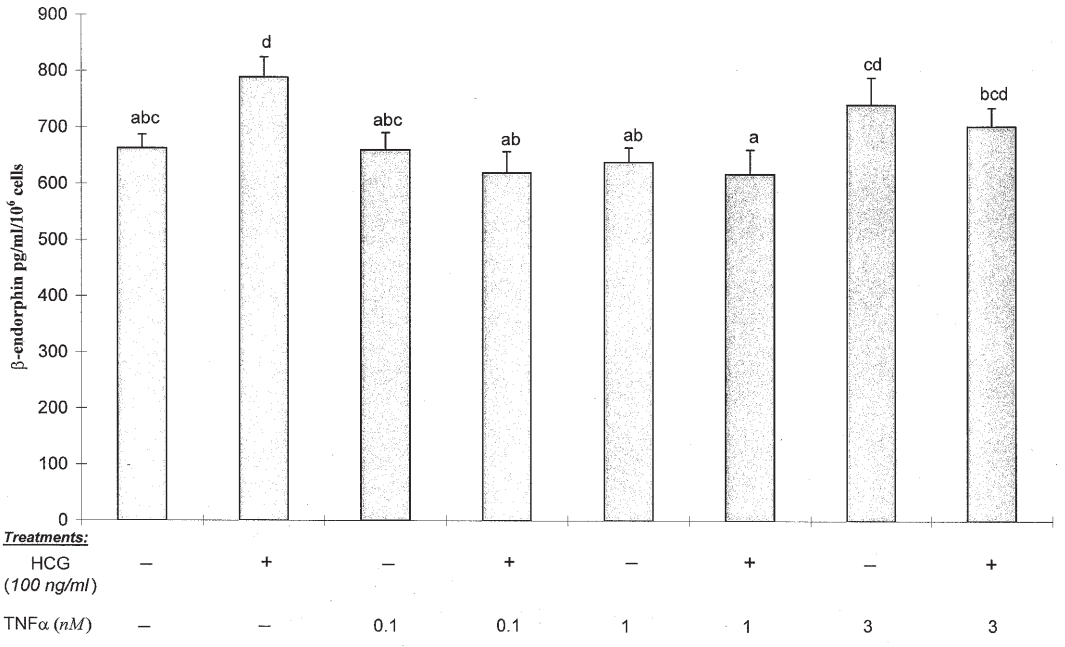

Fig. 3 Effect of hCG on β-END-LI secretion by porcine LLC in the absence or presence of tumor necrosis factor-α (TNFα). Results are means±S.E. of seven replications. Bars with different superscripts represent significantly different (P<0.05) values.

sion has been observed in experiments with granulosa cells (MELNER et al. 1986). On the other hand, it is well known that β-endorphin is involved in the regulation of GnRH-LH secretory system (MATSUSHITA et al. 1982; OKRASA 1997). Therefore, interactions between gonadotropins and β-endorphin within the hypothalamic-pituitary-ovarian axis are multidirectional.

TNFα is a 17 kDa cytokine produced within the corpus luteum, not only by macrophages, T lymphocytes and endothelial cells (Bogovandoss et al. 1988; Hehnke-Vagnoni et al. 1995), but possibly also by large luteal cells (Roby and Terranova 1989; Wuttke et al. 1993). For this reason TNFα, in addition to its other roles, is considered to be a potent local regulator of luteal function. The present results demonstrated that TNFα at doses of 1 nM and 0.1 nM inhibited hCG-induced, but not basal, β-END-LI secretion by LLCs, whereas it had no effect on β-endorphin secretion by SLCs. A similar action of TNFα was reported by Pitzel et al. (1993) and Richards and Almond (1994a) in relation to gonadotropin stimulated progesterone production by porcine SLCs. Additionally, Pitzel et al. (1993) observed an inhibitory influence of TNFα on basal steroidogenesis in both SLCs and LLCs of the pig. Studies by Richards and Almond (1994b) revealed the presence of TNFα binding sites on porcine luteal cell membranes. Although TNFα binding capacities were similar in small cell and large cell membranes, TNFα receptors on LLCs exhibited approximately 5-fold lower binding affinity than those on SLCs (Richards and Almond 1994b). Our results and those of Pitzel et al. (1993) imply that the lower-affinity TNFα binding sites present on LLCs are sufficient to mediate some effects of the cytokine. Taken together, it appears that both SLCs and LLCs are potential targets for TNFα within the porcine corpus luteum.

The mechanisms involved in TNFα interference with hCG induced β-endorphin secretion by LLCs are unknown. However, by analogy to ovarian theca-interstitial cells (Adashi et al. 1989; Zachow et al. 1993), TNFα might reduce the number of LH receptors in these cells. Furthermore, it has been shown that the mechanism of TNFα action may involve activation of protein kinase C (Zachow et al. 1992) and diminution of cAMP-dependent protein kinase A activity (Adashi et al. 1989). In the case of steroidogenesis these events may lead to inhibition of steroidogenic enzymes, e.g. 17α-hydroxylase/C17,20-lyase (Zachow and Terranova 1994). Wuttke et al. (1995) have reported that TNFα is capable of down-regulating the gene expression of several steroidogenic enzymes, including aromatase. The inhibitory effect of TNFα on hCG induced β-endorphin release from LLCs probably incorporates signaling pathways which are in part similar to those mediating the cytokine influence on gonadotropin regulated steroidogenesis, but this problem requires further elucidation.

Theoretically, the interrelation between TNFα and luteal β-endorphin described herein may have wider functional implications, since there is growing evidence of synergic effects of TNFα and prostaglandin $F_2\alpha$ in the initiation of corpus luteum regression in the pig (Wuttke et al. 1998). In our recent studies (Przala et al. 1998) we found increasing concentrations of β-END-LI in developing corpora lutea of the pig, with the highest values during the late luteal phase. Hence, it appears that β-endorphin may participate in local interactions controlling the life span of the porcine corpus luteum.

In summary, the present results clearly indicate that: 1. in the porcine corpus luteum LLCs are main source of β-endorphin, 2. hCG is capable of stimulating β-END-LI secretion by both LLCs and SLCs, 3. the effect of hCG on β-END-LI release from LLCs can be blocked by TNFα. Thus, luteal β-endorphin might be an important element of autocrine/paracrine regulation of corpus luteum function in pigs. Further studies are needed to elucidate the role of β-endorphin and TNFα interaction at different stages of the luteal phase.

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