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Original article

β-endorphin inhibition of progesterone secretion by porcine granulosa cells during follicle development

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Abstract – Exogenous β-endorphin or the opioid antagonist, naloxone, was added to the culture medium of control or LH-treated granulosa cells to elucidate the role of β-endorphin in follicular steroidogenesis. β-endorphin decreased basal progesterone (P_4) production by Gc isolated from small and medium follicles and had no effect on P_4 production by cells from large follicles. β-endorphin decreased P_4 production by LH-treated Gc isolated from small, medium and large follicles. Naloxone increased basal P_4 production by cells from small and medium follicles and had no effect on P_4 production by Gc from large follicles. Neither agent had any effect on basal oestradiol (E_2) secretion by Gc from any of the follicles. However, E_2 secretion by LH-treated Gc from all sizes of follicles was inhibited by β-endorphin, with the greatest effect on cells from small follicles. We conclude that opioid peptides act in the follicle in a paracrine manner to prevent excessive basal progesterone secretion by Gc and may also be involved in the regulation of LH action on granulosa cells steroidogenesis during follicle development. © Inra/Elsevier, Paris

opioid peptides / pig granulosa cells / follicular development / steroid secretion

Résumé – Effet inhibiteur de la β-endorphine sur la sécrétion de progestérone par des cellules de granulosa de truie. Afin d'étudier le rôle de la β-endorphine sur la stéroïdogenèse des cellules folliculaires, les effets de β-endorphine exogène ou de la naloxone (bloquant des récepteurs aux opioïdes) ont été évalués sur des cellules de granulosa. Un effet inhibiteur de la β-endorphine sur la sécrétion de progestérone (P_4) par les cellules de granulosa obtenues en début et milieu de cycle a été mis en évidence. Cet effet est absent quand des cellules de granulosa de follicules préovulatoires sont utilisées. En présence de LH, la β-endorphine réduit la sécrétion de progestérone par les trois types de granulosa étudiés. Un effet stimulateur de la naloxone sur la sécrétion de progestérone par les cellules de granulosa obtenues en début et

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milieu de cycle a également été mis en évidence, effet qui disparaît pour les cellules obtenues dans les follicules préovulatoires. En absence de LH, ni la β-endorphine ni la naloxone n'affectent la sécrétion d'œstradiol par les trois types de cellules de granulosa. Ces travaux démontrent le rôle important de la β-endorphine en tant que facteur prévenant une sécrétion excessive de progestérone par la granulosa et l'interaction existant entre la présence de LH et ces composés sur la stéréoïdogenèse. © Inra/Elsevier, Paris

opioïdes / granulosa / œstradiol / progestérone

1. INTRODUCTION

Regulation of reproductive mechanism is mediated in part by the action of opioid peptides, which may operate at multiple physiological levels. Recent studies have indicated that proopiomelanocortin (POMC)-mRNA-containing cells are present in antral follicles, corpus luteum and the interstitial compartment [16]. Opioid receptors have been detected in the uterus [1], placenta [20] and in Sertoli cell cultures [4, 7]. Although POMC-mRNA is present in the ovaries, and functional roles of POMC-derived peptides have been described [5, 8, 12, 19, 21], the relatively low concentration of opioid peptides in reproductive tissue suggests a predominant paracrine or autocrine role within the ovary [11, 13, 14]. Hamada et al. [11] demonstrated that the number of (^{3}[H]-naloxone)-specific binding sites in porcine granulosa cells decreases during follicular maturation. There are, however, no detailed studies on the direct effect of exogenous opioid peptides on steroidogenesis of granulosa cells dependent on follicular maturation. The aim of this study was to investigate the physiological role of β-endorphin in follicular development.

2. MATERIAL AND METHODS

2.1. Tissue preparation and culture

Porcine ovaries were obtained from a slaughterhouse. Granulosa cells (Gc) from small (4–6 days of the oestrus cycle), medium (10–12 days of the oestrus cycle) and large preovulatory (16–18 days of the oestrus cycle) follicles were isolated according to Stoklosowa et al. [18]. The first step in cell harvesting involved cutting the follicular wall with iris scissors. Granulosa cells were then obtained by scraping them off the follicular wall with a platinum loop. This process was repeated several times and the cells transferred to culture medium. Cells from freshly excised follicles from 3–5 animals at the same phase of the oestrous were pooled. The granulosa cell yield was 7.5×10^5 cells from small follicles, 1.3×10^6 cells from medium follicles and 1.5×10^6 cells from large follicles. The effect of β-endorphin (BE) on basal and LH-stimulated progesterone and estradiol production by these cells was then determined. Cells were plated (24-well plate, Nunc) in 1 mL fresh M199 medium containing 10 % calf serum and incubated at 37 °C (95 % air/5 % CO_2, 100 % humidity) overnight (16 h) with 1 000 ng BE ± 100 ng LH. Five doses of BE (0.1, 1, 10, 100 and 1 000 ng) were tested. The effect of the opioid peptide antagonist naloxone on progesterone and oestradiol production was investigated in cells incubated for 24 h with 100 μM naloxone ± 100 ng LH. The culture medium was assayed after 24 h for progesterone and oestradiol by RIA.

2.2. Steroid analysis

A radioimmunoassay was used for both progesterone and oestradiol [18].

2.2.1. Progesterone assay

A highly specific antibody directed against 11α-hydroxy-progesterone hemisuccinate, cou-

pled to bovine serum albumin (BSA) was raised in rabbits. The cross-reaction level with pregnenolone was 2.9 %. The other steroids tested showed a cross-reaction level below 1 %. (1,2,6,7-^{3}H) progesterone (Radiochemical Centre, Amersham, UK), was used as the tracer (80 Ci/mmol). The detection limit of the assay was 50 pg. The coefficient of variation of the assay was below 1.5 % and below 2.5 % between assays.

2.2.2. Oestradiol assay

A highly specific antibody directed against oestradiol 17β.-6-oxime-BSA antigen was raised in rabbits. It gave negligible cross-reaction with oestrone (0.8 %), oestriol (0.8 %) and 16-keto-oestradiol 17β (1 %). The other steroids tested gave a cross-reaction level below 0.001 %. (2,4,6,7,16,17-3H) oestradiol (Radiochemical Centre, Amersham, UK), was used as the tracer (140 Ci/mmol). The detection limit of the assay was 5 pg. The coefficient of variation of the assay was below 7.5 % and below 8 % between assays.

2.3. Statistical analysis

All data points are expressed as the mean ± SE, from at least three separate experiments (n = 3) each in triplicate. Significant differences in steroid concentration between control and experimental groups at different stages of follicular development, were compared by analysis of variance and Duncan's new multiple range test.

3. RESULTS

The dose response curve of progesterone and oestradiol secretion by granulosa cells isolated from follicles of the three different sizes under the influence of BE is given in *figure 1*. The greatest inhibitory effect on progesterone secretion was observed in cells from small ($P < 0.001$) and medium ($P < 0.05$) follicles to which 1 000 ng BE had been added. BE had no effect on oestradiol secretion by any of the three cell types investigated.

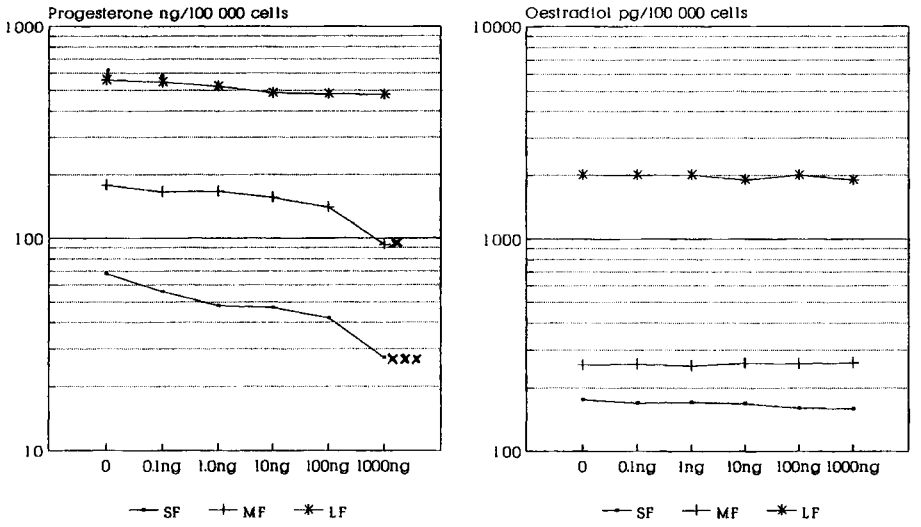


Figure 1. Dose response curve of progesterone and oestradiol secretion under the influence of β-endorphin. SF, small follicles; MF, medium follicles, LF, large follicles. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

3.1. Effect of β-endorphin (BE) on progesterone production by granulosa cells

In the absence of LH, 1 000 ng/mL BE significantly decreased progesterone production by cells isolated from small follicles (27.2 ± 0.71 ng versus 68.0 ± 1.41 ng/10^5 cells in control; $P < 0.001$) and medium follicles (117 ± 0.5 ng versus 178 ± 5.0 ng/10^5 cells in control; $P < 0.01$). It did not effect progesterone production by cells isolated from large follicles (*figure 2a*).

In the presence of 100 ng LH (LH-treated cells), BE significantly decreased progesterone production by cells isolated from small follicles (56.2 ± 0.71 ng versus 109 ± 0.5 ng/10^5 cells in cells in LH-treated cells; $P < 0.001$) whereas the effect of BE on cells isolated from medium (168.2 ± 5.6 ng versus 212.0 ± 3.5 ng/10^5 cells in LH-treated cells; $P < 0.01$) and large follicles (607.0 ± 4.8 ng versus 778 ± 3.9 ng/10^5 cells in LH-treated cells; $P < 0.01$) was less pronounced (*figure 2b*).

3.2. Effect of naloxone on progesterone production by granulosa cells

In the absence of LH, the addition of naloxone to the culture medium increased progesterone production by cells isolated from small (99.2 ± 2.1 ng versus 68.0 ± 1.41 ng/10^5 cells in control; $P < 0.01$) and medium (227 ± 5.9 ng versus 178 ± 5.0 ng/10^5 cells in control; $P < 0.01$) follicles but not large follicles (555 ± 1.5 ng versus 558 ± 2.5 ng/10^5 cells in control) (*figure 2a*). In the presence of LH the addition of naloxone had no effect on progesterone secretion by the three cell types investigated (*figure 2b*).

3.3. Effect of BE on oestradiol production by granulosa cells

The addition of BE to the culture medium had no effect on basal oestradiol secretion by the three cell types investigated (*figure 3a*). BE drastically decreased oestradiol secretion by LH-treated cells isolated from small follicles (87.5 ± 1.6 pg versus 170 ± 5.07 pg/10^5 cells in control; $P < 0.001$) and to a lesser but statistically significant extent by LH-treated cells isolated from medium (221.0 ± 2.7 pg versus 278 ± 3.1 pg/10^5 cells in control; $P < 0.01$) and large follicles (1 675 ± 5.7 pg versus 1 889 ± 1.9 pg/10^5 cells in control; $P < 0.01$) (*figure 3b*).

3.4. Effect of naloxone on œstradiol production by granulosa cells

The addition of naloxone to the culture medium had no effect on the basal oestradiol secretion by the cell types investigated, or on the synthesis of oestradiol by LH-treated cells (*figure 3a, b*).

4. DISCUSSION

Studies on β-endorphin synthesis and release during the oestrous cycle suggest that it exerts an autocrine or paracrine effect on the ovary [13, 14]. The effect of exogenous BE on progesterone and oestradiol production by granulosa cells isolated from small, medium and large preovulatory follicles was investigated in this study to obtain a clearer understanding of endogenous opioid peptides in follicular function. The action of the opioid antagonist naloxone was also analysed. The data indicate that β-endorphin decreases basal progesterone production by Gc isolated from small and medium follicles but has no effect on progesterone production by cells from large follicles. Naloxone increases basal progesterone production by cells from small and medium sized follicles but has no effect on progesterone production by Gc from large follicles. Data on POMC-mRNA-containing cells also suggest a paracrine role of opiopeptides in the ovary. Sanders et al. [16] showed

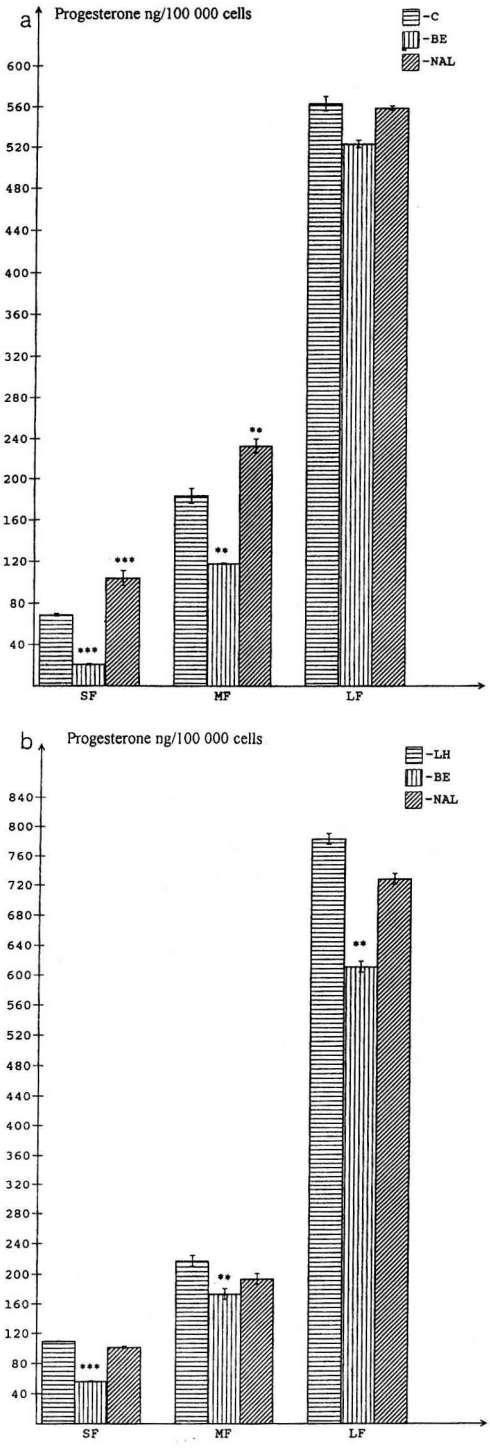
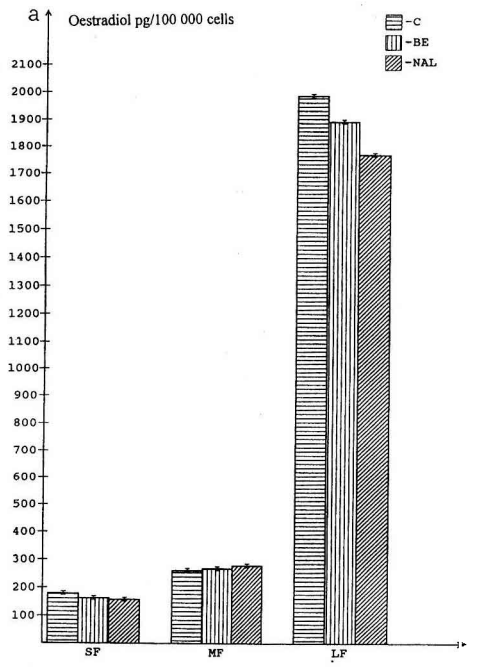


Figure 2. Effect of β-endorphin and naloxone on a) basal and b) LH-stimulated progesterone production by granulosa cells isolated from small (SF), medium (MF) and large (LF) follicles. Each bar represents the mean ± SE of nine replicate cultures. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.



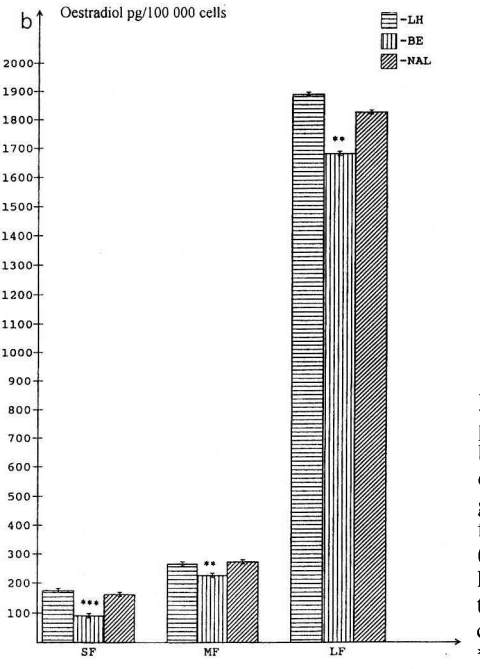


Figure 3. Effect of β-endorphin and naloxone on a) basal and b) LH-stimulated oestradiol production by granulosa cells isolated from small (SF), medium (MF) and large (LF) follicles. Each bar represents the mean ± SE of nine replicate cultures. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

that the number of POMC-mRNA-containing cells in the rat, increased during follicular development; however, their ovarian distribution suggested that the labelled cells could be non-endocrine, possibly white blood cells. A paracrine function of POMC-derived peptides in the rat testis has been suggested by Bardin et al. [2] and Chen et al. [6]. Gerendai et al. [9] suggested that β-endorphin inhibits Sertoli cell function during early testicular development. Bovine studies by Varsano et al. [21] suggested that opioid peptides may act as a 'switch' in steroidogenesis of the large luteal cells, preventing excessive basal progesterone synthesis. In contrast to results of Kato et al. [12] on rat luteal cells, we reported that in gilts BE alone alters ovarian steroidogenesis. This effect is only observed in small and medium follicles and is consistent with the decrease in number of specific ([^{3}H] naloxone) binding sites in porcine granulosa cells, during follicular maturation, demonstrated by Hamada et al. [11].

Our results are also consistent with the general view that the action of the opiate receptor antagonist naloxone is dependent on the gonadal steroid environment of the animal [3]. Opioid peptides may be involved in follicular development, in the modulation of gonadotropin action on the ovary or in the organization of the extracellular matrix. In LH-treated cells, BE decreases progesterone and oestradiol production by Gc isolated from all three follicular types. Since the addition of LH to the culture medium increased progesterone secretion by granulosa cells from all the follicles investigated, it is conceivable that the concentration of progestrone was sufficiently high to inhibit oestradiol secretion. Some authors [10, 17] have suggested that progesterone blocks follicular growth by a direct inhibition of oestradiol synthesis. Our observations support the suggestion of Sanders et al. [16] that opioid peptides are involved in follicular development for example in the modulation of gonadotropin action. It is conceivable that steroid hormones act in concert with opioid neuropeptides as modulators of the steroidogenic effect of other hormones, such as LH or FSH. The decrease in basal progesterone secretion by granulosa cells isolated from small and medium sized follicles and in progesterone secretion by LH-treated cells, suggests that intra-ovarian opioids have a paracrine effect responsible for the gradual transformation of follicles from the early stages of development to maturation. Further studies are required to elucidate the underlying mechanism.

In conclusion, our data support the relationship between porcine folliculogenesis and ovarian opioid peptides suggested by Hamada et al. [11], and suggest that opioid peptide is involved in the regulation of action of LH on granulosa cell steroidogenesis during follicular development.

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