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INVOLVEMENT OF HIGH-DENSITY LIPOPROTEIN IN STIMULATORY EFFECT OF HORMONES SUPPORTING FUNCTION OF THE BOVINE CORPUS LUTEUM

D. SKARŻYŃSKI, J. MŁYNARCZUK and J. KOTWICA*

Institute of Animal Reproduction and Food Research, Polish Academy of Sciences, 10-718 Olsztyn-Kortowo, Poland

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The hypothesis that epinephrine (noradrenaline, NA) enhances utilisation of high-density lipoproteins (HDL) by bovine luteal cells and that this process involves phospholipase (PL) C and protein kinase (PK) C intracellular pathway was tested. Luteal cells from days 2–4, 5–10 or 11–17 of the oestrous cycle were preincubated for 20 h. Subsequently DMEM/Ham's F-12 medium was replaced by fresh medium and the cells were treated for 6 h as follows: In Experiment I with HDL (5–75 μg cholesterol per ml), NA, isoprenaline (ISO) or luteinising hormone (LH). In Experiment II cells were incubated for further 24 h in deficient medium (without FCS) and next treated as in Experiment I. In Experiment III cells were stimulated with NA, ISO or LH alone and together with HDL. In Experiment IV cells were treated with PLC inhibitor (U-73122) or with PKC inhibitor (staurosporine) or stimulator (phorbol 12-myristrate 13-acetate) and with either NA, insulin or LH. Only luteal cells from days 5–10 of the cycle responded on HDL and β-mimetics ($P < 0.05$). LH stimulated progesterone secretion from the luteal cells during all stages of the cycle ($P < 0.001$). Cells incubated in deficient medium and supplemented with HDL secreted as much progesterone as those stimulated by LH in all stages of the cycle. Beta-mimetics were unable to enhance the stimulatory effect of HDL. Blockade of PLC had no influence on progesterone secretion from cells treated with either NA or LH, but this did impair the stimulatory effect of insulin ($P < 0.05$). Similarly, blockade of PKC by staurosporine impaired ($P < 0.05$) the effect of insulin only but not that observed after LH or NA treatment. We suggest that: (a) noradrenergic stimulation does not enhance utilisation of cholesterol from HDL for progesterone secretion; (b) the fasting of luteal cells seems to activate enzymes responsible for the progesterone synthesis; (c) effect of NA on progesterone secretion from luteal cells does not involve the PLC-PKC pathway.

Key words: Cattle, corpus luteum, HDL, progesterone, PKC

It is reported that noradrenaline (NA) increases secretion of progesterone and ovarian oxytocin from bovine corpus luteum (CL) both *in vivo* (Kotwica et al., 1991*b*; Kotwica and Skarzynski, 1993) and *in vitro* (Miszkiel and Kotwica,

*Corresponding author; E-mail: janko@pan.olsztyn.pl; Fax: +48 (89) 524 0347

2001) studies. This stimulatory effect of NA is through the activation of β-receptors of luteal cells (Kotwica et al., 1991*a*) and further through the activation of specific enzymes involved in the progesterone synthesis (Miszkiel and Kotwica, 2001). Thus the effect of NA supports CL function, and this support seems to be very important especially during short-lasting stressful situations in pregnant females as suggested by Kotwica and Bogacki (1999). It is also known that NA enhances lipolysis and ovarian blood flow (Wiltbank et al., 1990). The ovary uses the cholesterol from high-density lipoproteins (HDL) as a substrate source for steroid hormone production (Williams, 1989) and addition of lipoproteins stimulated synthesis of progesterone in cultured luteal cells (Pate and Condon, 1989; Carroll et al., 1992). Therefore we assumed that under the influence of NA, the CL may receive higher amounts of cholesterol with HDL as a source for progesterone synthesis. To verify this hypothesis, dispersed luteal cells were treated with β-mimetics and luteinising hormone (LH). In our model variable blood flow through the CL was mimicked by the different doses of HDL. Moreover, it was found that NA stimulates $PGF_{2\alpha}$ secretion from early and mid-luteal cells (Skarzynski et al., 2000), which further increases progesterone secretion, activating phospholipase C (PLC) activity and the protein kinase C (PKC) intracellular pathway (Davis et al., 1987; Okuda et al., 1998). Hence we wanted to study whether luteotropic hormones can affect the progesterone secretion through this intracellular pathway.

Materials and methods

Corpora lutea collection and preparation of cells

Ovaries with corpus luteum (CL) were collected in a commercial slaughterhouse within 30 min after killing of cyclic cows. Corpora lutea from days 2–4, 5–10 or 11–17 of the oestrous cycle of cows were collected as described by Ireland et al. (1980). Ovaries were transported to the laboratory in container with saline on ice-bath. Luteal cells were obtained by CL perfusion with mixture of collagenase IA (1 mg/ml) and DNase I (5 μg/ml) as described by Skarzynski and Okuda (2000). Cells obtained from 2–3 CL being at the same stage of the cycle were pooled and their viability was estimated by means of 0.04% Trypan Blue exclusion. Luteal cells (10^5/ml) suspended in 0.5 ml of DMEM and supplemented with 10% FCS were placed into 48-well plates (Corning Inc., USA) and pre-incubated for 24 h to attach cells to bottom of well. Thereafter the medium was changed, cells were washed twice with M-199 (Sigma) containing 0.1% BSA. Then 0.5 ml of DMEM supplemented with 0.1% BSA, ascorbic acid (20 μg/ml), transferrin (5 μg/ml), and sodium selenite (5 μg/ml, ICN) were added and cells were cultured in atmosphere of air containing 5% of CO_2 at 100% humidity, at 37 °C (Heraus BB-6060, Germany). All media were enriched with

gentamycin (20 µg/ml; ICN). Luteal cells for each individual experiment were pooled from 2–3 CL and each treatment was done in 3 or 4 replications. All other chemicals used in these studies were purchased from Sigma.

Experiment 1

To define the effective dose of HDL cells were treated for 6 h with this lipoprotein (5–75 µg cholesterol per ml). Obtained results were compared to those caused by NA (10^{-5}M), isoprenaline (ISO; 10^{-5}M) and LH (100 ng/ml), used as positive control. After end of incubation medium was collected and frozen (–20 °C) until assay.

Experiment 2

After 24 h pre-incubation cells were further incubated for 24 h in deficient medium (0.1% BSA). Later these cells were treated as in Experiment 1.

Experiment 3

Luteal cells were stimulated for 6 h with NA, ISO and LH together with HDL (10 µg of cholesterol per ml) after pre-incubation as given in Experiments 1 and 2.

Experiment 4

Cells were treated for 6 h with NA, insulin (1 µg/ml) or LH and with either phospholipase (PL)C inhibitor (U-73122; 10^{-5}M) or protein kinase (PK)C inhibitor (staurosporine). Moreover, the intracellular mechanisms of luteotropic agents were evaluated in PKC-deficient luteal cells next treated with either NA or insulin or LH. These cells were produced by pre-incubation for 24 h with chronic exposure to PKC stimulator (phorbol 12-myristrate 13-acetate; PMA; 10^{-6} M).

Analysis

Progesterone content in medium was determined by enzyme immunoassay (EIA; Okuda et al., 1998). Progesterone antiserum (R-1; kindly donated by Dr. S. Okrasa of University of Warmia and Mazury, Olsztyn) was used at a final dilution of 1:75,000. The standard curve ranged from 0.39 to 100 ng/ml and the effective dose for 50% inhibition (ID 50) of the assay was 4.5 ng/ml. The intra- and interassay coefficients of variation were 5.9% and 8.9%, respectively.

Statistical analysis

The data are presented as the mean (± SEM) of values obtained from 4–6 CL each performed in 3–4 repetitions. The statistical significance of differences values in groups was assessed by ANOVA and Newman-Keuls post-test (GraphPad, PRISM, Software, San Diego, CA, USA).

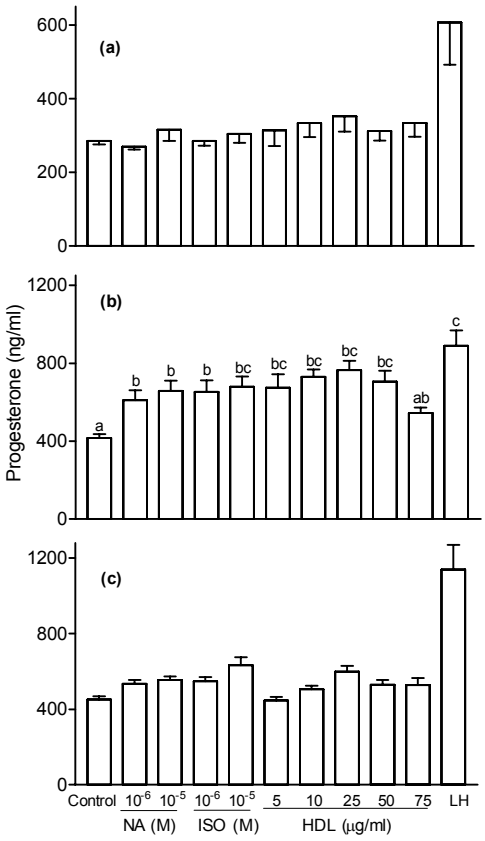

Fig. 1. Influence for 6 h of noradrenaline (NA), isoprenaline (ISO) and different doses of HDL (5–75 μg cholesterol per ml) on progesterone secretion by luteal cells from days 2–4 (a), 5–10 (b) and from days 11–17 (c) of the oestrous cycle. Cells were pre-incubated for 24 h in 10% FCS medium before treatment. [a,b,c]Values with different superscripts are significantly different ($P < 0.05$)

Results

The effect of used factors on luteal cell function was dependent on the stage of the oestrous cycle. After 6-h incubation only luteal cells from days 5–10 of the cycle responded significantly ($P < 0.05$) on HDL and on β-mimetics treatment (Fig. 1b). On basis of these data 10 μg of cholesterol was used in Experiment 3. LH stimulated P4 secretion during all stages of the cycle ($P < 0.001$) and the potency of this effect increased ($P < 0.05$) toward the end of the cycle. However, cells incubated in deficient medium for 24 h and next supplemented with HDL secreted as much progesterone as those stimulated by LH in all stages of the cycle ($P < 0.01$; Fig. 2). On days 2–4 of the cycle HDL stimulated progesterone more evidently ($P < 0.05$) than did LH (Fig. 2a). These cells incubated in de-

ficient medium and next supplemented with HDL released higher amount of progesterone (Fig. 2a) compared to those secreted by luteal cells from the later days of the cycle. Beta-mimetics stimulated progesterone secretion ($P < 0.05$) from cells on day 5–10 of the cycle when given alone (Fig. 3a) but they were unable to enhance the effect observed after HDL supplementation (Fig. 3a). Blockade of PLC had no influence on progesterone secretion from cells treated with NA or LH, but this did impair ($P < 0.05$) the effect of insulin (Fig. 4a). Blockade of PKC by staurosporine impaired effect of insulin ($P < 0.05$) only but not that evoked by LH or NA (Fig. 4b). Similar profiles of progesterone secretion were observed from PKC-deficient cells (Fig. 4c).

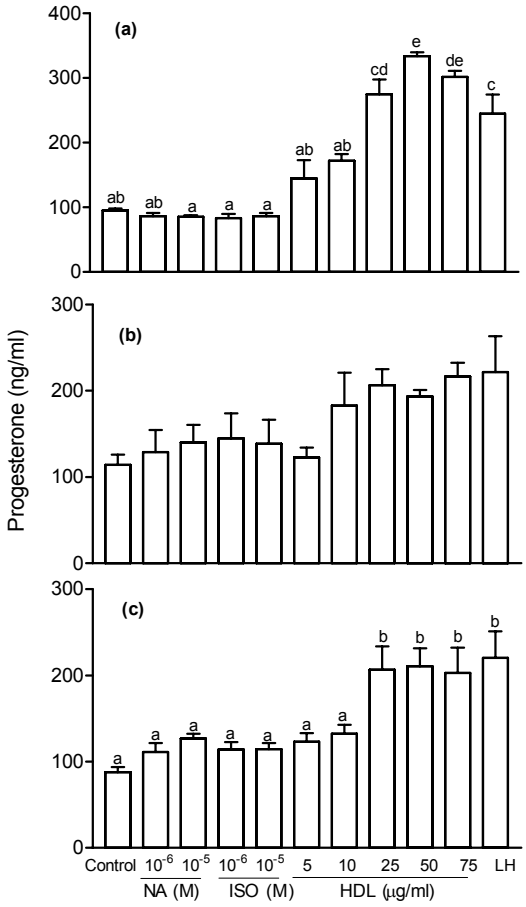

Fig. 2. Effect for 6 h of noradrenaline (NA), isoprenaline (ISO) and HDL (5–75 μg cholesterol per ml) on progesterone secretion by luteal cells from days 2–4 (a), 5–10 (b) and from days 11–17 (c) of the oestrous cycle. Before treatment cells were pre-incubated for 24 h in 10% FCS medium and thereafter incubated for next 24 h in deficient medium supplemented with 0.1% of BSA only.
[a,b,c]Values with different superscripts are significantly different ($P < 0.05$)

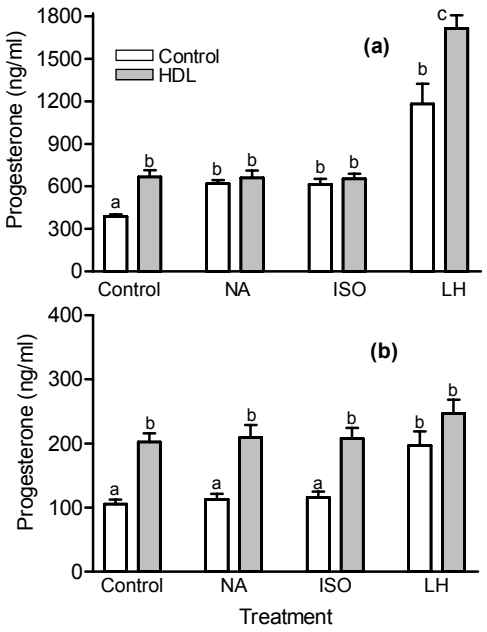

Fig. 3. Effect of noradrenaline (NA), isoprenaline (ISO) and LH on progesterone secretion by luteal cells from days 5–10 of the oestrous cycle for 6 h (a); or before treatment cells were pre-incubated for 24 h in deficient medium supplemented with 0.1% of BSA only (b). Cells were supplemented or not with HDL (10 µg of cholesterol per ml). [a,b,c]Values with different superscripts are significantly different ($P < 0.05$)

Discussion

Doses of HDL used in these studies (equivalent of 5–75 µg cholesterol per ml) are within the ranges used by Pate and Condon (1989) and Carroll et al. (1992) in similar studies. These amounts of cholesterol increased progesterone secretion from cells on days 5–10 of the oestrous cycle in our study. It was found earlier that the receptor-mediated uptake is the primary method of importing cholesterol for the synthesis of progesterone (Strauss et al., 1984) and luteal cells have specific B, E receptors which bind apolipoproteins associated with lipoproteins (Goldstein and Brown, 1977). Moreover, Hwang and Menon (1983) discovered that these receptors are regulated by gonadotropins. Since in our study HDL supplementation increased progesterone release by luteal cells from days 5–10 of the cycle only (Figs 1b and 3a), it is assumed that very early and late luteal cells have less B, E receptors. It is worthy to underline that sensitivity of luteal cells to LH stimulation in these studies increases with the age of CL as it was observed by Baird (1992). Supplementation of cells incubated in deficient medium with HDL increased progesterone release by about 200% on days 2–4 and

by 100% on later days of the oestrous cycle (Figs 2 and 3b). This effect of HDL on progesterone secretion from luteal cells is comparable with that caused by LH in the same medium conditions. These results would mean that luteal cells have a broad area of functional autonomy independently of their subordination to the central regulations. It should be noticed that basal secretion of progesterone by cells cultured in deficient medium was 2–4 times lower compared to the cells incubated with FCS-enriched medium. Our observations allow us to speculate that shortage of cholesterol as a substrate for steroidogenesis in luteal cells up-regulates the activity of enzymes involved in the progesterone synthesis. This assumption helps to explain the similar secretion of progesterone by fasted cells supplemented with HDL or with LH. Furthermore, LH enhanced stimulatory effect of HDL whereas NA and ISO (β-mimetic) did not (Fig. 3a). Since both LH and NA increase the activity of enzymes involved in progesterone synthesis (Carlson et al., 1971; Miszkiel and Kotwica, 2001), these data suggest that LH can also support the penetration of lipoproteins into the luteal cells. It has been shown that NA acting through the β-adrenergic receptor (Kotwica et al., 1991*b*; Miszkiel and Kotwica, 2001) may regulate $PGF_{2\alpha}$ receptor-PLC-PKC cascade in cultured bovine luteal cells (Skarzynski et al., 2000). Moreover, some modulating interactions between the PKA- and PKC-signal transduction pathways have been well established in many cell types (Cohen, 1988; Houslay, 1991) including bovine luteal cells (Mamluk et al., 1999). However, we did not confirm the hypothesis that NA can affect progesterone secretion from bovine CL directly via PLC-PKC intracellular pathway. However, it is known that the bovine CL contains a number of different isoforms of PKC (Orwig et al., 1994*a, b*) and further studies are necessary to elucidate which of them may interact with PLA-PKA intracellular pathway. Contrary to these data, insulin could stimulate progesterone secretion activating both PLC and PKC since blockade of this pathway diminished stimulatory influence of this hormone. High-affinity insulin binding sites have been detected in the membranes from the bovine luteal tissue (Sauerwein et al., 1992). Insulin binding sites belong to the protein tyrosine kinase (PTK) receptor family (Chakravorty et al., 1993; Liu and Wang, 1994). Increase of activity of this type receptor is associated with activation of several signal transduction enzymes including PLC-PKC intracellular pathway (Liu and Wang, 1994; Blume-Jansen, 1997). Thus, contrary to the β-adrenergic receptor, activation of insulin receptor PTK-mediated pathways may be manifested by PKC-dependent progesterone secretion in the bovine luteal cells, as shown in Fig. 4.

Based on the obtained data we assume that: (a) HDL as a source of cholesterol increased progesterone secretion within 6 h but noradrenergic stimulation of luteal cells did not enhance the effect evoked by HDL; (b) shortage of cholesterol within CL does appear to up-regulate the activity of enzymes responsible for progesterone synthesis; (c) contrary to insulin, NA affecting the secretion of progesterone from luteal cells does not act through the pathway of PKC.

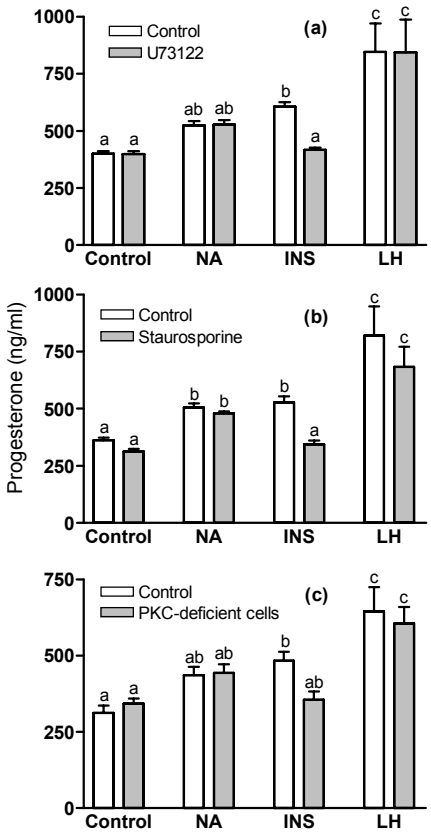

Fig. 4. Influence of noradrenaline (NA), insulin (INS) and LH on luteal cells from day 5–10 of the oestrous cycle. Concomitantly with these hormones blocker of phospholipase C (PLC) – U-73122 (10^{-6} M) (a), or staurosporine (10^{-6} M) inhibitor of protein kinase C (b) was given. PKC-deficient cells were obtained by incubation of cells for 24 h with phorbol 12-myristate 13-acetate (10^{-6} M) (c). [a,b,c]Values with different superscript are significantly different (at least $P < 0.05$)

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