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Rapid communication

Up-regulation of lymphocytic growth hormone secretion during the luteal phase of cycle and early pregnancy

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ABSTRACT

Growth hormone (GH) has been shown to be produced and secreted by priphereal immune cells. Therefore, we studied the release of GH by lymphocytes, during various stages of pregnancy and estrous cycle in the cow. The effect of leptin on the lymphocytic GH release was also investigated. Estradiol-17β and progesterone concentrations in plasma were measured in all animals to confirm their reproductive status. Growth hormone levels measured in cell cultures during early pregnancy (days 60–80) and during the luteal phase were greater ($p \leq 0.01$) than levels during follicular phase or mid (days 100–160) and late (days 240–245) pregnancy. Leptin treatment stimulated ($p \leq 0.05$) lymphocytic GH release during mid-pregnancy when the basal GH levels were low. Changes in lymphocytic GH release and elevation of lymphocytic GH secretion by leptin during pregnancy and the absence of such effects in estrous cycle may indicate that leptin modulation of lymphocytic GH plays a role in the regulation of immune response during pregnancy.

1. Introduction

Lymphocytes are increasingly viewed as autocrine or paracrine organs [1] capable of supplying specific tissues with a vast array of substances including classical hormones such as adrenocorticotropin (ACTH) and growth hormone (GH) to modulate cellular (immune) functions [2]. Growth hormone binding sites have been shown on immune cells [3], and GH is involved in the development and regulation of immune system [4,5]. It modulates the migration of developing T cells [6] and improves the leucocytes' function in ovariectomized old rats [7]. Further, GH enhances thymic function in HIV-1-infected men [8]. Taken together, there is a close relationship between GH and immune system.

Dixit and Parvizi, [9] reported increased production of ACTH from cattle peripheral blood mononuclear cells (PBMC; in the following will be addressed to as lymphocytes) in early pregnancy. Adrenocorticotropin has mostly immunosuppressive actions [10], whereas GH exerts predominantly immunostimulatory effects [11]. Thus, the balance in the expression of ACTH and GH in lymphocytes might be critical during pregnancy and other conditions in which the immune system is activated.

Therefore, the present study was designed to evaluate the lymphocytic GH secretion in various stages of the pregnancy and during estrous cycle in the cow. As a first step towards the elucidation of the regulatory mechanisms, we tested the effects of leptin. We used leptin because, our previous study showed that leptin induces GH secretion from porcine lymphocytes [12], and that leptin binds to the lymphocytes which contain GH [12].

2. Materials and methods

2.1. Animals

A total of 30 healthy lactating Holstein Friesian cows from the research farm of the Institute for Animal Genetics, Neustadt, Germany, were used. The animals were randomly divided into the following groups, according to their reproductive status: 1) Early-pregnancy (days 60–80 after artificial insemination, AI; age = 2.9 ± 0.49 years; lactation, lac = 1.7 ± 0.4; milk yield, my = 7070 ± 973 l; $n = 6$), 2) Mid-pregnancy (days 100–160 AI; age = 4.4 ± 0.4 years; lac = 2.1 ± 0.3; my = 7685 ± 512 l; $n = 6$), 3) Late-pregnancy (days 240–245 AI; age = 3.0 ± 0.6 years; lac = 2.0 ± 0.4; my = 7491 ± 736; $n = 5$), 4) Follicular phase of estrous cycle (0–1 days prior to the expected estrus; age = 3.6 ± 0.6 years; lac = 1.7 ± 0.5; my = 7879 ± 728 l; $n = 4$), 5) Luteal phase of estrous cycle (6–12 days after estrus; age = 3.6 ± 0.4 years; lac = 1.7 ± 0.3; my = 7894 ± 663 l; $n = 9$). Age, lactation and milk yield were not significantly different among the groups. Eight animals (2 from group 1; 1 from group 4 and 4 from group 5) were sold or moved to another station before their milk yield could be recorded. The animals were milked twice daily at 05.00 h and

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14.40 h. They received a mixed ration (Inst. For Animal Nutrition, FAL, Braunschweig, Germany) balanced to meet their individual requirements, and had free access to water.

The experimental herd of the institute is supervised by veterinarians for control of the health and general condition of the animals. The animals are milked up to approximately 6 weeks prior to the estimated day of parturition. The insemination occurs at 3rd or 4th estrus (50–70 days) post partum. The estrus is routinely diagnosed by an experienced technician controlling the external signs of estrus (changes in appearance of vulva and behavior) daily. Plasma estradiol-17β (E2) and progesterone concentration were measured to confirm the reproductive status of the animals at the experimental day. The pregnancy is diagnosed using trans rectal sonography on day 45 AI. The body score of the animals was not recorded in this experiment, however, the body score of lactating HF cows in this herd is 2.5 to 3 (on a 1 to 5 scale, Velazquez unpublished data).

Blood samples were collected in EDTA containing tubes by jugular venipuncture for harvesting of the lymphocytes and measurement of E2 and progesterone. The study was conducted from October to April when the animals were kept indoors. The study was conducted in accordance with the procedures approved by the animal ethics committee.

2.2. Preparation of lymphocytes

The lymphocytes were separated as described previously [9]. The EDTA mixed blood was centrifuged at 2600 rpm for 20 min at 20 °C, the buffy coat was harvested and re-suspended in Hanks' balanced salt solution (HBSS, Sigma, Steinheim, Germany). Buffy coats were carefully layered on a lymphocyte separation medium, Lymphodex (Inno-Train, GmbH, Germany), and centrifuged again. The ring of lymphocytes was sucked out, and the cells were re-suspended and washed four times with HBSS. Contaminating erythrocytes were lyzed through a hypotonic shock by washing in double-distilled water. Thereafter, the lymphocytes were washed again with HBSS.

2.3. Cell culture

The lymphocytes were re-suspended in a solution consisting of 1:1 RPMI-1640 (Roswell Park Memorial Institute-1640; Sigma, Steinheim, Germany) medium and HBSS. This RPMI-1640 and HBSS cell culture medium was supplemented with 1% fetal calf serum (Sigma), 1% antibiotic/antimycotic mixture (Sigma) containing 10.000 IU Penicillin, 10 mg streptomycin and 25 μg amphotericin per ml. The cell count was adjusted at 1×10^6 per ml. The cells were seeded in four-well culture plates (Nunc Brand Product, Darmstadt, Germany) and incubated for 48 h at 37.2 °C and 5% CO_2. At the beginning of the incubation some of the cell cultures were treated with Leptin (100 nM, Sigma). We used this dose, because our previous work showed that this is the effective dose in porcine lymphocytes [12]. Cell viability was tested by trypan blue exclusion test and cell preparations with more than 95% viability were cultured.

2.4. Gel chromatography

At the end of 48 h of incubation, media from three plates ($4\times3=12$ wells) of control incubation or treatments were pooled and centrifuged at 2000 rpm for 10 min at 20 °C. The medium was then eluted for GH by gel chromatography using 1.5×30 cm Sephadex G-50 fine column. The column was equilibrated with 0.1 M PBS containing 5% bovine serum albumin, and 26 aliquots of 1.5 ml each were collected and stored at −20 °C. These samples were subsequently lyophilized using beta-1 lyophilizer (Christ, Osterode, Germany). Then reconstituted in 300 μl of assay buffer (0.01M PBS, 0.025M EDTA, 0.25% BSA, 0.01% thimerosal; pH 7.4) for GH assay.

2.5. Hormone assay

Growth hormone was measured in duplicate by a radioimmunoassay according to the method described previously [13] and adapted for GH measurements in cell culture medium [13]. The recovery of highly purified bovine GH (Biogenesis, Dorset, UK) added to the culture medium was 99.2 ± 1.21%. All reagents were diluted in assay buffer. The lower limit of detection (10% displacement) was 0.5 ng/ml. Half maximum displacement was achieved at 6 ng/ml. The intra and inter-assay coefficients of variation were 7.5% and 13% respectively. GH was measured in all 52 aliquots (26×2) of each animal. All samples from one animal were analyzed in the same assay.

Plasma E2 and progesterone concentrations were measured using enzyme-immunoassays as described previously [9]. The cross-reaction of the E2 antiserum was <2%, <0.3%, <0.004%, <0.0001% and <0.0001% with estrone, estriol, testosterone, progesterone and cortisol, respectively. The inter- and intra- assay coefficient of variation was 16.6% and 11.7%, respectively.

The cross-reaction of progesterone antiserum with E2, dihydrotestosterone, testosterone and 17-alpha hydroxyprogesterone was <0.1%. The sensitivity of the assay was 12.5 pg/ml. Inter- and intra-assay coefficients of variation did not exceed 3.3% and 10%, respectively.

2.6. Statistical analysis

For statistical evaluations, GH values measured in aliquots 7–16 of each 26 aliquots were pooled, thus, each animal was presented with one value in each treatment group. The results are expressed as mean ± SEM and were statistically analyzed by one-way ANOVA followed by Tukey's test for comparisons between groups employing SPSS software. Student's *t*-test was used for comparisons of the control and treatment values in each group. A probability of $p\leq0.05$ was considered to be statistically significant.

3. Results

Plasma estradiol-17β and progesterone levels were monitored in all animals to confirm the stage of the cycle and pregnancy. Estradiol-17β concentration was greatest ($P\leq0.05$) during the follicular phase and on days 240–245 of pregnancy (Table 1). Plasma progesterone values were greatest ($P\leq0.05$, Table 1) during early pregnancy (days 60–80).

GH concentration was greater ($P\leq0.01$) in media from lymphocyte cultures from luteal phase and days 60–80 of pregnancy than from the follicular phase of the estrous cycle, and days 100–160 and 240–245 of pregnancy (Fig. 1). A significant decline ($P\leq0.001$) in GH values was observed (Fig. 1) between early pregnancy (days 60–80) and mid-pregnancy (days 100–160). The levels remained low until the end of pregnancy (Fig. 1).

An increase ($P\leq0.05$) in GH levels was observed when the lymphocytes harvested from cows during mid-pregnancy (days 100–160) were incubated with leptin (Fig. 2). In contrast, leptin treatment during the luteal phase of the estrous cycle or the early pregnancy (days 60–80) as well as during the late pregnancy (days 240–245) had no effects (Fig. 2).

Table 1
Plasma estradiol-17β (pg/ml; $\bar{x}\pm$SEM) and progesterone (ng/ml; $\bar{x}\pm$SEM) during various phases of estrous cycle and pregnancy in the cow.

	Estrous cycle		Pregnancy		
	Follicular phase (4)[1)]	Luteal phase (9)	days 60–80 (6)	days 100–160 (6)	days 240–245 (5)
Estradiol-17β	12.6 ± 2.5^{a}	1.8 ± 0.47^{b}	1.9 ± 0.35^{b}	5.4 ± 0.54^{c}	11.7 ± 0.80^{a}
Progesterone	1.0 ± 0.10^{a}	5.0 ± 0.67^{b}	8.0 ± 1.1^{c}	5.4 ± 0.41^{b}	5.4 ± 1.3^{b}

Means with different letters within a row differ significantly (at least $P\leq0.05$).
[1)] Number of animals.