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CELL BIOLOGY

Roles of Leptin and Ghrelin in the Regulation of the Phenotype and Cytokine Production by NK Cells from Peripheral Blood

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Abstract—Both leptin and ghrelin used separately at the concentrations corresponding to trimesters II–III of pregnancy increase the number of $CD56^{bright}$ NK cells in mononuclear cell suspension; their combination also enhances the L-selectin expression on the surface of these cells in the culture. These hormones do not affect the production of TGF-β1, IL-17A, or IFN-γ by NK cells, and they inhibit the production of IL-10. Leptin decreses the IL-4 production by $NKp46^{+}$ cells, but the presence of ghrelin abrogates this effect.

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Leptin and ghrelin are peptide hormones. They are functional antagonists in regulation of feeding behavior, adipose tissue metabolism, energy metabolism, and immunoreactivity [1]. Simultaneous action of leptin and ghrelin at both the cell body levels results in cooperative effects that determine a balanced energy level and homeostasis of immune responses [1]. Lipid metabolism and immunoreactivity change substantially during pregnancy due to growth of a fetus [2, 3]. Both hormones are generated by the placenta and fetus tissues and play an important role at all gestation stages, controlling implantation, growth, and development of the fetus [1]. Systemic inhibition of adaptive immunity during pregnancy is compensated by the activation of innate immunity, the most important effectors of which are natural killer cells (NK cells). NK cells are the most abundant cell population among lymphoid cells in decidual membrane during early pregnancy stage [4]. Decidual $CD56^{bright}$ NK cells have no cytotoxic activity and secrete large amounts of cytokines, which participate in implantation and neoangiogenesis [4]. More than 80–90% of mature $CD16^{+}CD56^{dim}$ NK cells in peripheral blood display higher cytotoxicity compared to $CD16^{-}CD56^{bright}$ lymphocytes [4]. The cytolytic potential of the NK cell pool decreases during pregnancy due to a decrease in the percentage of $CD16^{+}CD56^{dim}$ lymphocytes; an increase in their concentration in peripheral blood is associated with a refractory pregnancy loss [4]. It is known that $CD56^{bright}$ NK cells are able to produce different cytokines. This fact is at the basis of distinguishing separate subsets of NK cells. For example, NK1 cells produce predominantly IFN-γ and tumor necrosis factor (TNF-α); NK2 cells, interleukins IL-4, IL-5, IL-6, and IL-13; NK3 cells, transforming growth factor (TGF-β); NKr1, and IL-10 [5, 6].

The presence of inhibitory receptors, especially NKG2A, on the cell surface is an important parameter of functional activity of NK cells. The NKG2A expression increases in the first weeks of pregnancy and reaches its maximum by the third month of gestation [7]. It has been shown in several studies that leptin and ghrelin play an important role in regulation of the functional activity of NK cells that express specific hormone receptors [8–10].

The aim of our study was to explore the roles of leptin and ghrelin at the concentrations characteristic of pregnancy in regulation of the expression of surface molecules and cytokines production by NK cells in peripheral blood of women in vitro.

Suspensions of isolated peripheral blood mononuclear cells (PBMCs) of healthy nonpregnant women at fertile age from 23 to 38 years were used. PBMC suspensions were obtained by centrifugation in ficoll–verografin density gradient with $\rho = 1.077$ g/cm^3. The cells were cultivated in complete culture medium (CCM) consisting of RPMI 1640 medium, 10% fetal calf serum (Sigma-Aldrich, United States), 10 mM Hepes (ICN Pharmaceuticals, United States), 2 mM L-glutamine (ICN Pharmaceuticals, United States), 100 μg/mL gentamicin (KRKA, Slovenia), and hormones leptin (Sigma-Aldrich, United States) and ghrelin (Euroscreen, Belgium). Leptin was used at concentrations of 10 and 35 ng/mL reflecting its concentration in peripheral blood during trimesters I and

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Table 1. The effects of leptin, ghrelin, and their combination on NKG2A and L-selectin (CD62L) expression by isolated NK cells ($M \pm m$, $n = 7$)

Group	$NKp46^+NKG2A^+$, %	$CD56^{bright}CD62L^+$, %	$CD56^{dim}CD62L^+$, %
Control	21.67 ± 1.31	8.31 ± 0.77	27.01 ± 4.63
Leptin, 10 ng/mL	21.55 ± 3.47	9.58 ± 2.80	29.85 ± 4.96
Leptin, 35 ng/mL	21.48 ± 2.66	12.47 ± 1.90*	32.63 ± 5.30
Ghrelin, 1.25 ng/mL	22.36 ± 3.98	10.48 ± 2.07	30.75 ± 5.07
Ghrelin, 0.83 ng/mL	24.10 ± 4.76	11.28 ± 2.23	32.34 ± 6.13
Leptin, 35 ng/mL + ghrelin, 0.83 ng/mL	24.73 ± 2.32	10.79 ± 0.61*	31.49 ± 4.02

Here and in Table 2 and the figure, * $p < 0.05$ as compared to control.

II–III of pregnancy, respectively [3]. Ghrelin was used at concentrations of 1.25 and 0.83 ng/mL, which are comparable with its concentrations in peripheral blood during trimesters in I–II and III of pregnancy, respectively [2].

For studying simultaneous action, the hormones were added to culture at the concentrations that caused statistically significant effects. Specifically, leptin was used at a concentration of 35 ng/mL and ghrelin, 0.83 ng/mL. The same sterile physiological saline in which the hormones had been dissolved was added to control samples instead of the hormone solutions. PBMC suspensions were incubated with the hormones for 72 h for the assessment of the hormone influence on CD56 expression, which was determined by monoclonal antibody Anti-human CD56-PE (Beckman-Coulter, United States). The NK cell phenotype was determined afterwards. In these series of experiments, the cells were identified on the basis of the presence of the specific NKp46 marker, which was detected by Anti-human CD335-PC5 (Beckman Coulter, United States), in lymphocytic gate by FACS analysis. The NKp46 molecule is constitutively present on the surface of all NK cell population, and it is one of the main markers used for their identification [11].

At the next stage, NK cells were selected from PBMC suspension by immunomagnetic isolation using the Dynabeads Untouched Human NK Cells kit (Invitrogen, United States). The isolation purity judged by the NKp46 marker expression was 95%. The enriched suspension of $NKp46^+$ immunopositive cells at a concentration of 5×10^6 cells/mL was incubated in 500 μL of CCM with hormones for 72 h at 37°C in an atmosphere with 5% of CO_2. Cytokines (Gibco, United States) IL-2 at a concentration of 1 ng/mL, IL-12 at a concentration of 2 ng/mL, and IL-15 at a concentration of 10 ng/mL were added to all samples to support viability of isolated NK cells [12]. The NK cell phenotype was assessed after the incubation by FACS in lymphocytic gate. CD56 expression, L-selectin expression, which was detected by Anti-human CD62L-FITC (Beckman Coulter, United States), and the presence of NKG2A inhibitory molecules, which was detected by Anti-human CD159-PE (Beckman Coulter, United States), on the cell surface were assessed in the isolated NK cells. The staining of the cells was performed according to the protocol of the manufacturer of the monoclonal antibodies. No less than 100 000 cells were counted. The corresponding isotype controls were used for the control of nonspecific binding and the allocation of fluorescence-negative lymphocytic gate. The concentrations of IL-4, IL-10, IL-17, INF-γ, and TGF-β1 were measured in the cell culture supernatants by ELISA (Tsitokin, Russia and R&D, United States).

Since the distribution of the obtained data in all experimental groups was normal, as judged by the Kolmogorov–Smirnov test, the significance of the differences between the groups was assessed by paired Student's *t* test.

As mentioned above, $CD56^{bright}$ NK cells predominantly fulfill immunoregulatory functions, thus providing a favorable progress of pregnancy. We revealed (figure) that leptin and ghrelin at the concentrations characteristic of trimesters II–III of pregnancy significantly increase $NKp46^+CD56^{bright}$ cell counts in PBMC suspension. Taking into account that leptin and ghrelin are functional antagonists [1], we additionally performed the estimation of their simultaneous action in those concentrations, which exerted statistically significant effect. We found that simultaneous addition of leptin and ghrelin to the incubation medium in concentrations characteristic of trimesters II–III of pregnancy also increase significantly the $NKp46^+CD56^{bright}$ cell counts. We did not observe an additive effect or a synergism (figure). Similar conversion of isolated from PBMC NK cells to $NKp46^+CD56^{bright}$ cells was described upon incubation of NK cells with TGF-β1 or decidual stroma cells [13]. Thus, it may be stated that the peptide hormones studied are factors of endocrine control that promote expression of CD56 molecules on the surface of NK cells during the late period of pregnancy.

Next stage of the study consisted in the assessment of the leptin and ghrelin effects on the expression of L-selectin and NKG2A in the isolated NK cell popu-

Table 2. Hormone action on cytokines secretion by isolated NK cells ($M \pm m$, $n = 9$)

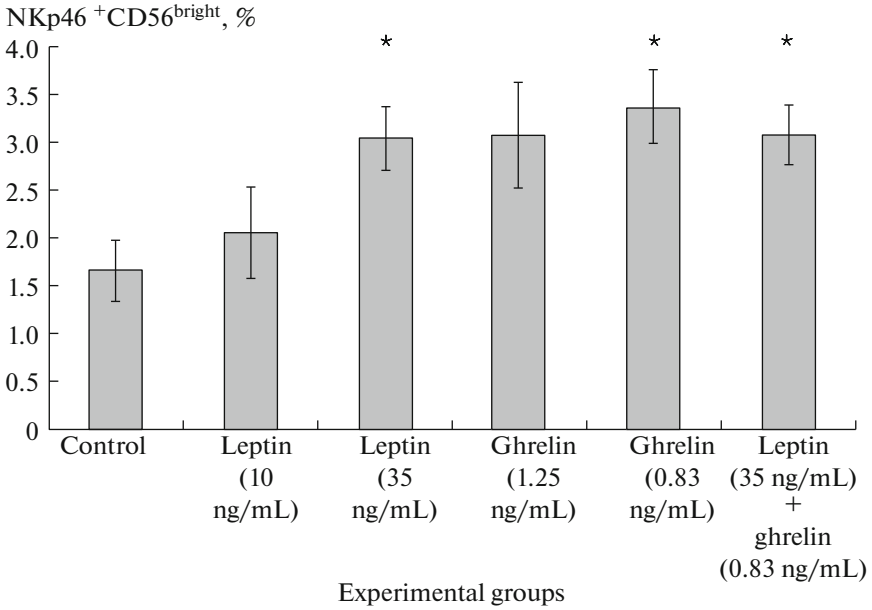
Group	IL-4	IL-10	TGF-β1	IL-17A	IFN-γ
Control	8.69 ± 0.19	25.62 ± 1.25	263.08 ± 7.84	239.36 ± 4.74	506.45 ± 26.40
Leptin, 10 ng/mL	7.57 ± 0.98	24.96 ± 1.69	270.49 ± 20.37	225.39 ± 12.38	520.45 ± 45.29
Leptin, 35 ng/mL	7.63 ± 0.31*	23.61 ± 0.97*	275.88 ± 19.99	221.63 ± 13.63	555.91 ± 36.44
Ghrelin, 1.25 ng/mL	8.87 ± 0.89	23.89 ± 1.95	271.98 ± 10.39	253.35 ± 14.39	450.36 ± 45.69
Ghrelin, 0.83 ng/mL	8.26 ± 0.58	22.04 ± 1.33*	267.50 ± 8.00	244.58 ± 15.85	439.89 ± 55.32
Leptin, 35 ng/mL + ghrelin, 0.83 ng/mL	8.29 ± 0.83	22.20 ± 1.30*	271.07 ± 8.94	213.00 ± 11.12	444.15 ± 52.47

lation. The high-affinity inhibitory receptor NKG2A (a ligand for NKG2A is HLA-E, which is a "nonclassical" molecule of histocompatibility complex I) does not allow NK cells to lyse recognized trophoblastic cells [14], while L-selectin (CD62L) expression on $CD56^{bright}$ NK cells is associated with their migration to placental zone [15].

We revealed that only leptin at the concentration typical of the second half of pregnancy increased the number of $CD56^{bright}$ NK cells bearing L-selectin (Table 1). Leptin and ghrelin did not significantly affect the expression of L-selectin or NKG2A on the outer membrane of $CD56^{dim}$ NK cells. Similar results were obtained in the study of a combination of hormones of the second half of pregnancy. Probably, only the stimulatory effect of leptin takes place in this case. Note that leptin-induced stimulation of the surface molecules studied occurred only in the $CD56^{bright}$ subpopulation of NK cells. Leptin significantly increased the number of cells in this subpopulation. Apparently, these two processes are interconnected and regulated by leptin in the second half of the pregnancy. This fact is important because it shows the possibility of hormone-driven late migration of $CD56^{bright}$ NK cells to the decidual part of the placenta.

It is known that $CD56^{bright}$ NK cells are able to produce large amounts of cytokines. NK1 cells producing IFN-γ predominate in peripheral blood of nonpregnant women [5]. The number of NKr1 cells secreting IL-10 increases during pregnancy. Decidual cells are mainly represented by NK3 lymphocytes expressing TGF-β1, which determines their immunosuppressive effect on many lymphocyte populations [5].

Studying the cytokine spectrum of NK cells upon treatment with leptin and ghrelin, we found (Table 2) that both hormones affected the production of IFN-γ, TGF-β1, and IL-17A, but inhibited the production of IL-10 by $NKp46^{+}$ cells irrespective of the hormone concentrations. In addition, leptin at a high concentration also decreased the production of IL-4 by iso-



The effects of leptin, ghrelin, and their combination on $NKp46^{+}CD56^{bright}$ cell counts in PBMC suspension in vitro (percent, $n = 12$).

lated NK cells. The combination of the hormones characteristic of the second half of pregnancy also did not affect the production of IL-17A, IFN-γ, or TGF-β1, but significantly decreased the IL-10 production by $NKp46^+$ NK cells. However, we did not observe the inhibitory effect of leptin in combination with ghrelin on IL-4, which indicated the opposite effect of ghrelin compared to leptin, neutralizing the effect of the latter.

The peptide hormones studied do not affect the production of the cytokines that determine the type of regulatory NK cells, such as NK1 and NK3, but they reduce the probability of NK2 and NKr1 subtype formation. However, the effect of these hormones is possible only in trimesters II–III of pregnancy, and this physiological aspect needs further investigation.

In general, it may be stated that leptin and ghrelin, as well as their combination, at the concentrations characteristic of the late period of pregnancy, partly regulate the expression of membrane molecules and cytokine production by NK cells in vitro. It is possible that the hormones studied augment endocrine regulation of NK cells during physiological pregnancy in its late period.

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