EFFECT OF THE GROWTH HORMONE-RELEASING HORMONE [GHRH(1-44)NH_2] ON IL-6 AND IL-8 SECRETION FROM HUMAN PERIPHERAL BLOOD MONONUCLEAR CELLS IN VITRO

A. SIEJKA, T. STEPIEN, H. LAWNICKA, R. KRUPINSKI, J. KOMOROWSKI, H. STEPIEN

Department of Endocrinology, Medical University of Lodz, 91-425 Lodz, Poland
e-mail: hstep@csk.am.lodz.pl

Objective. Bidirectional communication between the neuroendocrine and immune systems is now a subject of an intensive investigation. Growth hormone-releasing hormone (GHRH) is synthesized by the hypothalamus, but is present also in the immune cells. Some recent data indicate also an immunomodulatory role of the neuropeptide. The aim of the study was to examine the influence of GHRH(1-44)NH_2 on interleukin-6 and interleukin-8 secretion from human peripheral blood mononuclear cells cultured in vitro.

Methods. Peripheral blood mononuclear cells (PBMC) were isolated by centrifugation using Böyum technique and cultured in a humidified atmosphere of 5 % CO_2 and 95 % O_2 at 37 ^{0}C for 24 hours in the presence of lipopolysaccharide (LPS) at the concentration of 2 μg/ml and GHRH(1-44)NH_2 (the final neuropeptide concentrations to be tested were 10^{-12} to 10^{-6} M). ELISA methods were used to measure IL-6 and IL-8 concentrations in the supernatants of cultured cells

Results. GHRH(1-44)NH_2 influenced IL-6 secretion from cultured cells, but significant inhibition of IL-6 release was observed at 10^{-6} M ($p<0.001$). The negative correlation between the GHRH concentration studied and the IL-6 level in the supernatants was found ($r = -0.759$; $p<0.001$). GHRH had no influence on the secretion of IL-8 from activated PBMC.

Conclusions. Our results demonstrate that GHRH in vitro modulates IL-6 secretion from the human peripheral blood mononuclear cells, without any significant effect on IL-8 secretion.

Key Words: Growth hormone – releasing hormone – Interleukin-6 – Interleukin-8 – Neuroimmunomodulation

Recent studies clearly indicate close bidirectional communication between the neuroendocrine and the immune systems, and hypothalamic releasing hormones, apart from their neuroendocrine role, has been shown to influence immune function (BLALOCK 1994; PAWLIKOWSKI et al. 1994; SPANGELO and GOROSPE 1995; WEIGENT and BLALOCK 1995). Growth hormone-releasing hormone (GHRH) is produced not only by neurosecretory cells of hypothalamic nuclei, but is also synthesized by immunocompetent cells (KHORRAM et al. 2001; STEPHANOU et al. 1991; WEIGENT and BLALOCK 1990; WEIGENT et al., 1991) and may modulate their function acting possibly via GHRH-receptor (GUARCELLO et al., 1991).

Interleukin 6 (IL-6), produced by several cells including monocytes/macrophages, T cells and B cells, is a cytokine that plays a central role in the defense mechanism regulation, acute phase reaction and hematopoiesis (AKIRA et al. 1990; HIRANO et al. 1992; VAN SNICK 1990). Some modulatory effects of IL-6 on the neuroendocrine system have been demonstrated (SPANGELO and GOROSPE 1995; ARTZ et al. 1998; GLODDEK et al. 2001; THIELE et al. 2003). Overproduction of IL-6 is associated with a spectrum of age-related conditions, including cardiovascular diseases, type 2 diabetes and certain cancers (KIECOLT-GLASER et al. 2003).

Interleukin 8 (IL-8), a member of CXC subfamily of chemokines, is also produced by numerous types of cells (including monocytes/macrophages, T cells, neutrophils, fibroblasts, endothelial cells, keratinocytes, hepatocytes, astrocytes, chondrocytes) (DUNLEVY et al. 1995) in response to proinflammatory stimuli such as

lipopolysaccharide (LPS), interleukin 1 (IL-1) and cachectin (TNFα) (Baggiolini et al. 1995). It is a potent neutrophil chemotactic and activating factor (Watanabe et al. 1989; Collins et al. 1991). Il-8 is also a modulator of angiogenesis and tumour growth (Koch et al. 1992; Masood et al. 2001; Moore et al. 1998; Inoue et al. 2000). Depending on the cells source, at least four N-terminal variants of human IL-8 containing 79, 77, 72, or 69 amino-acids have been described. Both the 72 amino-acid (also referred to as the leukocyte form) and 77 amino-acid (endothelial-derived form) forms of IL-8 show similar biological activities.

So far, the effect of GHRH on IL-6 and IL-8 secretion from PBMC has remained unknown. Thus, the aim of the study was to evaluate the influence of somatoliberin (GHRH(1-44)NH_2) on IL-6 and IL-8 secretion from human peripheral blood mononuclear cells cultured in vitro.

Materials and Methods

Subjects. Four healthy adult volunteers (in order to determine IL-6 secretion) and eight healthy adult volunteers (for IL-8 secretion determination) (men; age:27-30 yrs) were examined in this study. Blood samples (40 ml) were taken between 08.00-09.00 a.m., with the subjects having fasted overnight.

Cell culture preparation. The peripheral blood mononuclear cells (PBMC) were isolated from the freshly heparinized blood by density centrifugation with Lymphoprep (Nyegaard & Co. A/S, Oslo, Norway), according to a technique described by Böyum (Böyum 1968). The cells were washed twice with RPMI-1640 medium containing 10 % fetal calf serum (FCS, Hungarpol) and gentamycin (Krka, SLO) (1 μg/ml). Cell numbers were determined by light microscopy count, and viability was assessed by the trypan blue dye exclusion technique. Then, the cells were suspended in RPMI-1640 medium with 10% heat inactivated FCS and distributed in 700 μl aliquots per disposable 24 well tissue culture plates (Nunclon Multidish 24 wells, NUNC, Denmark) at the final concentration of 2×10^6 cells/ml. The cells cultured at 37 °C in a humidified atmosphere of 95 % of air and 5 % of CO_2 were stimulated by the suboptimal dose (2 μg/ml) of lipopolysaccharide (LPS, Sigma, USA) and after 2 hours of preincubation, the growth hormone-releasing hormone [GHRH(1-44)NH_2, Sigma, USA] (at the final concentrations 10^{-12}, 10^{-10}, 10^{-8} and 10^{-6} M) was added. The equal volume of culture medium was added to the appropriate wells (in order to obtain the final volume of 1 ml in each culture well).

Twenty hours after the addition of tested peptides, incubation was stopped and samples of supernatants, after centrifugation, were collected and frozen at -80 °C until IL-6 and IL-8 determination.

Human IL-6 and IL-8 ELISAs (R&D Systems, USA) were used to measure IL-6 and IL-8 concentrations, respectively (lower levels of assay sensitivities were: <0.7 pg/ml and 3.5 pg/ml, respectively. Intra-assay and inter-assay precisions were <4.4 % and <3.7 % for Quantikine IL-6 immunoassay, and <4.7 % and <8.1 % for Quantikine IL-8 immunoassay respectively. The Quantikine IL-8 immunoassay is based on antibodies raised against the 72 amino acid variant of human IL-8 derived from *E. coli* and it is calibrated with the same recombinant factor.

Statistical evaluation. All results are expressed as means ± SEM. Comparisons between tested groups were made by Student's t test. The differences were considered significant at $p<0.05$. For the obtained IL-6 concentrations, Pearson's index was calculated (r) and correlation's significance was measured with t-Student's test.

Results

Cell viability in the presence of GHRH was not significantly different from that observed in control cultures (estimated by trypan blue dye uptake after 24 hours of cell cultures incubation).

GHRH alone (without LPS) had no influence on IL-6 secretion [data not shown]. The suboptimal dose of LPS significantly stimulated IL-6 production ($p<0.05$) (Tab. 1). GHRH(1-44)NH_2 influenced IL-6 secretion into the supernatants of cultured cells and statistically significant inhibition was observed at high concentration of 10^{-6} M (Tab. 1). In addition, significant negative correlation between the GHRH concentration and IL-6 secretion was found ($r=-0.759$; $p<0.001$) (Fig. 1).

As expected, the suboptimal dose of LPS significantly stimulated IL-8 secretion ($p<0.01$), but GHRH(1-44)NH_2 did not modulate the secretion of IL-8 from the activated cells (Fig. 2).

Discussion

Our results indicate that GHRH is able to influence IL-6 secretion. GHRH inhibited IL-6 secretion from

Table 1
The effect of human GHRH (1-44)NH_2 at 10^{-12} to 10^{-6} M concentrations on IL-6 levels in supernatants of PBMC cultured in vitro (mean ± SEM)

	IL-6 concentration [pg/ml]		
	Mean (Xe)	**±SEM**	**P**
Control (C)	3878.5	103.4936	–
Control +LPS (C+LPS)	39155	375.0889	P<0.001 vs C
GHRH 10^{-12}M	39495	1192.738	NS vs C+LPS
GHRH 10^{-10}M	38895	301.7035	NS vs C+LPS
GHRH 10^{-8}M	37155	1068.968	NS vs C+LPS
GHRH 10^{-6}M	34935	178.5824	P<0.001 vs C+LPS

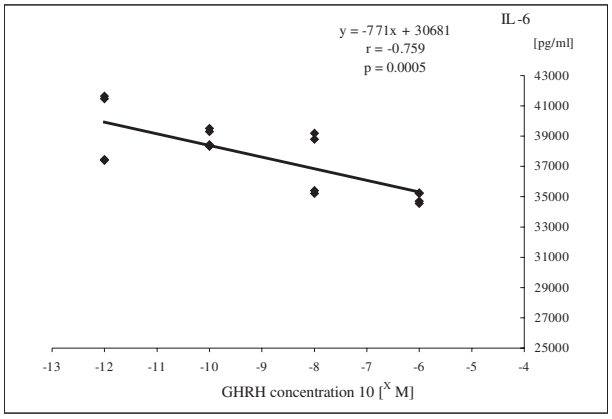

Fig 1 Correlation between GHRH(1-44)NH_2 concentrations and IL-6 levels measured in the supernatants of cultured cells (r = -0.759; p<0.001).

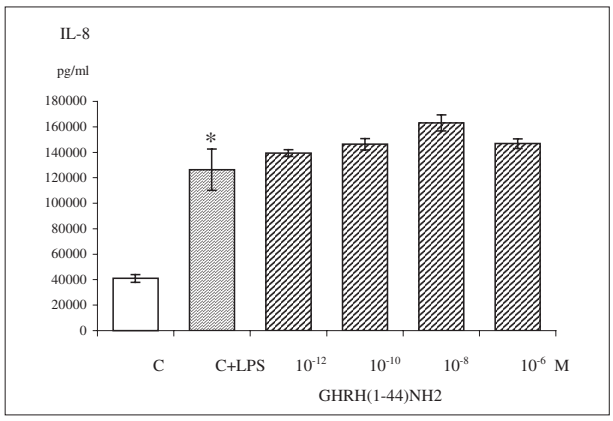

Fig 2 The effect of human GHRH (1-44)NH2 at 10-12 to 10-6 M concentrations on IL-8 levels in supernatants of PBMC cultured in vitro (bars represent mean ± SEM; C-control; LPS- lipopolysaccharide; * p<0.01 vs C). The results present the pooled data from eight separate cell cultures (4 wells for each concentration in a single experiment).

PBMC with statistical significance observed at 10^{-6} M of GHRH. The effect of GHRH on IL-6 secretion is opposite to that observed after its functional antagonist (somatostatin, SRIF) employment. Thus, in the study of Komorowski and Stepien (1995) SRIF in concentration from 10^{-8} M to 10^{-10} M significantly potentiated the release of IL-6 from LPS activated cultured monocytes. GHRH, tested in the same concentration range, did not change IL-8 secretion from PBMC in the present study, whereas Komorowski et al. (2000) demonstrated inhibitory effect of somatostatin and octreotide on IL-8 secretion from human PBMC.

The present and previous studies demonstrate that GHRH modulates some, but not all functions of the immune system. It has been shown that the synthetic human GHRH inhibits the chemotactic response of PBMC (Zelazowski et al. 1998). The natural killer (NK) activity of peripheral blood lymphocytes was suppressed by GRF at the concentrations of 10^{-10} to 10^{-6} M, but only when an effector : target cell ratio of 40:1 is used. In contrast, at effector : target ratios of 20:1 and 10:1, stimulatory effects of GRF were observed (Pawlikowski et al. 1988). GHRH also modulated interleukin-2 (IL-2) and interferon-gamma (IFN-γ) secretion from human immunocytes (Valtorta et al. 1991; Khorram et al. 1997; Siejka et al. 2004).

GHRH mRNA expression in peripheral blood mononuclear cells (PBMC) was significantly lower in the postmenopausal than in the premenopausal women and was not different in the PBMC of young men compared with old men (Khorram et al. 2001). Treatment with GHRH analogue in aging men and women (mean age of 66.9 yrs) resulted in potent activation of the T lymphocyte, monocytes and B cell function within 1 month (Khorram et al. 1997). In transgenic mice

which overexpress the human GHRH, splenocytes had a significantly higher proliferative activity (Blazar et al. 1995; Dialynas et al. 1999). In patients with growth hormone deficiency, (GHD) increased levels of IL-6 concentrations have been found (Leonsson et al. 2003). In mice IL-6 inhibited growth hormone-releasing factor (mGHRF) secretion by cultured placental cells and the antibodies to IL-6 or IL-6 receptors completely blocked the inhibitory effect of the IL-6 on mGHRF secretion (Yamaguchi et al. 1995).

In conclusion, our study demonstrates modulatory effect of human GHRH on certain functions of the immune system. Thus, we show for the first time that GHRH inhibits IL-6 secretion from human peripheral blood mononuclear cells in vitro, without any significant effect of GHRH on IL-8 release.

Acknowledgement

The study was supported by grants from the Medical University of Lodz No 502-11-724/92 and 502-11-187 (to H.S.)

References

Akira S, Hirano T, Taga T, Kishimoto T: Biology of multifunctional cytokines: IL 6 and related molecules (IL 1 and TNF). FASEB J **4**, 2860-2867, 1990

Artz E, Paez Pereda M, Costas M, Sauer J, Renner U, Holsboer F, Stalla GK: Cytokine expression and molecular mechanisms of their auto/paracrine regulation of anterior pituitary function and growth. Ann N Y Acad Sci **840**, 525-31, 1998

Baggiolini M, Loetscher P, Moser B: Interleukin-8 and the chemokine family. Int J Immunopharmacol **17**, 103-108, 1995

Blalock JE: The syntax of immune-neuroendocrine communication. Immunol Today **15**, 504-511, 1994

Blazar BR, Brennan CA, Broxmeyer HE, Shultz LD, Vallera DA: Transgenic mice expressing either bovine growth hormone (bGH) or human GH releasing hormone (hGRH) have increased splenic progenitor cell colony formation and DNA synthesis in vitro and in vivo. Exp Hematol **23**, 1397-406, 1995

Böyum A : Isolation of mononuclear cells and granulocytes from human blood. Isolation of mononuclear cells by one centrifugation and of granulocytes by combining centrifugation and sedimentation at 1g. Scand J Clin Lab Invest **21** (suppl 97), 77-89, 1968

Collins PD, Jose PJ, Williams TK: The sequential generation of neutrophil chemoattractant proteins in acute inflammation in the rabbit in vivo. Relationship between C5a and proteins with the characteristics of IL-8/neutrophil-activating protein 1. J Immunol **146**, 677-684, 1991

Dialynas E, Brown-Borg H, Bartke A: Immune function in transgenic mice overexpressing growth hormone (GH) releasing hormone, GH or GH antagonist. Proc Soc Exp Biol Med **221**, 178-183, 1999

Dunlevy JR, Couchman JR: Interleukin-8 induces motile behavior and loss of focal adhesions in primary fibroblasts. J Cell Sci **108**, 311-321, 1995

Gloddek J, Lohrer P, Stalla J, Artz E, Stalla GK, Renner U: The intrapituitary stimulatory effect of lipopolysaccharide on ACTH secretion is mediated by paracrine-acting IL-6. Exp Clin Endocrinol Diabetes **109**, 410-5, 2001

Guarcello V, Weigent DA, Blalock JE: Growth hormone releasing hormone receptors on thymocytes and splenocytes from rats. Cell Immunol **136**, 291-302, 1991

Hirano T: Interleukin 6 and its regulation of inflammation and disease. Clin Immunol Immunopathol **62**, 60-65, 1992

Inoue K, Slaton JW, Eve BY, Kim SJ, Perrotte P, Balbay D, Yano S, Bar-Eli M, Radinsky R, Pettaway CA, Dinney CPN: Interleukin 8 expression regulates tumoregicity and metastases in androgen-independent prostate cancer. Clin Cancer Res **6**, 2104-2119, 2000

Khorram O, Yeung M, Vu L, Yen SS: Effects of [norleucine27]growth hormone-releasing hormone (GHRH) (1-29)-NH2 administration on the immune system of aging men and women. J Clin Endocrinol Metab **82**, 3590-3596, 1997

Khorram O, Garthwaite M, Golos T: The influence of aging and sex hormones on expression of growth hormone-releasing hormone in the human immune system-1. J Clin Endocrinol Metab **86**, 3157-3161, 2001

Kiecolt-Glaser JK, Preacher KJ, MacCallum RC, Atkinson C, Malarkey WB, Glaser R: Chronic stress and age-related increases in the proinflammatory cytokine IL-6. Proc Natl Acad Sci USA **100**, 9090-9095, 2003

Koch AE, Polverini PJ, Kunkel SL, Harlow LA, DiPietro LA, Elner VM, Elner SG, Strieter RM: Interleukin-8 as a macrophage-derived mediator of angiogenesis. Science **258**, 1798-1801, 1992

KOMOROWSKI J and STEPIEN H: Somatostatin (SRIF) stimulates the release of interleukin-6 (IL-6) from human peripheral blood monocytes (PBM) in vitro. Neuropeptides **29**, 77-81, 1995

KOMOROWSKI J, JANKIEWICZ-WIKA J, STEPIEN H : Somatostatin (SOM) and octreotide (OCT) inhibit the secretion of interleukin-8 (IL-8) from human peripheral blood mononuclear cells (PBMC) in vitro. Horm Metab Res **32**, 337-338, 2000

LEONSSON M, HULTHE J, JOHANNSSON G, WIKLUND O, WIKSTRAND J, BENGTSSON BA, OSCARSSON J: Increased interleukin-6 levels in pituitary-deficient patients are independently related to their carotid intima-media thickness. Clin Endocrinol (Oxf) **59**, 242-50, 2003

MASOOD R, CAI J, TULPULE A, ZHENG T, HAMILTON A, SHARMA S, ESPINA BM, SMITH DL, GILL PS: Interleukin 8 is an autocrine growth factor and a surrogate marker for Kaposi's sarcoma. Clin Cancer Res **7**, 2693-2702, 2001

MOORE BB, ARENBERG DA, ADDISON CL, KEANE MP, POLVERINI PJ, STRIETER RM: CXC chemokines mechanism of action in regulating tumor angiogenesis. Angiogenesis **2**, 123-134, 1998

PAWLIKOWSKI M, STEPIEN H, KOMOROWSKI J: The hypothalamic-pituitary-thyroid axis and the immune system. Neuroimmunomodulation **1**, 149-152, 1994

PAWLIKOWSKI M, ZELAZOWSKI P, DOHLER K, STEPIEN H: Effects of two neuropeptides, somatoliberin (GRF) and corticoliberin (CRF), on human lymphocyte natural killer activity. Brain Behav Immun **2**, 50-56, 1988

SIEJKA A, LAWNICKA H, KOMOROWSKI J, STEPIEN T, KRUPINSKI R, STEPIEN H: Effect of growth hormone-releasing hormone (GHRH) and GHRH antagonist (MZ-4-71) on interferon-γ secretion from human peripheral blood mononuclear cells in vitro. Neuropeptides **38**, 35-39, 2004

SPANGELO BL and GOROSPE WC: Role of the cytokines in the neuroendocrine-immune system axis. Front Neuroendocrinol **16**, 1-22, 1995

STEPHANOU A, KNIGHT RA, LIGHTMAN SL: Production of growth-hormone-releasing hormone-like peptide and its mRNA by human lymphocytes. Neuroendocrinology **53**, 628-633, 1991

THIELE JO, LOHRER P, SCHAAF L, FEIRER M, STUMMER W, LOSA M, LANGE M, TICHOMIROWA M, ARTZ E, STALLA GK, RENNER U: Functional in vitro studies on the role and regulation of interleukin-6 in human somatotroph pituitary adenomas. Eur J Endocrinol **149**, 455-61, 2003

WATANABE K, KINOSHITA S, NAKAGAWA H: Purification and characterizaton of cytokine-induced neutrophil chemoattractant produced by epithelioid cell line of normal rar kidney (NRK-52E cell). Biochem Biophys Res Commun **161**, 1093-1099, 1989

WEIGENT DA and BLALOCK JE: Immunoreactive growth hormone-releasing hormone in rat leukocytes. J Neuroimmunol **29**, 1-13, 1990

WEIGENT DA and BLALOCK JE: Associations between the neuroendocrine and immune sustems. J Leukoc Biol **58**, 137-50, 1995

WEIGENT DA, RILEY JE, GALIN FS, LEBOEUF RD, BLALOCK JE: Detection of growth hormone and growth hormone-releasing hormone-related messenger RNA in rat leukocytes by the polymerase chain reaction. Proc Soc Exp Biol Med **198**, 643-648, 1991

VALTORTA A, MORETTA A, MACCARIO R, BOZZOLA M, SEVERI F: Influence of growth hormone-releasing hormone (GHRH) on phytohemagglutinin-induced lymphocyte activation: comparison of two synthetic forms. GHRH and PHA-induced lymphocyte activation. Thymus **18**, 51-59, 1991

VAN SNICK J: Interleukin-6: an overview. Annu Rev Immunol **8**, 253-278, 1990

YAMAGUCHI M, MIKI N, ONO M, OHTSUKA C, DEMURA H, KURACHI H, INOUE M, ENDO H, TAGA T, KISHIMOTO T, et al.: Inhibition of growth hormone-releasing factor production in mouse placenta by cytokines using gp130 as a signal transducer. Endocrinology **136**, 1072-8, 1995

ZELAZOWSKI P, DOHLER KD, STEPIEN H, PAWLIKOWSKI M: Effect of growth hormone-releasing hormone on human peripheral blood leukocyte chemotaxis and migration in normal subjects. Neuroendocrinology **50**, 236-239, 1989

Corresponding author: Prof. Henryk Stepien, M.D., Ph.D.
Institute of Experimental Endocrinology,
Medical University of Lodz
91-425 Lodz, Sterling 3, Poland
Phone/Fax: +48-42-632 4854
E-mail: hstep@csk.am.lodz.pl