AGE ASSOCIATED VARIATIONS IN LYMPHOCYTIC GROWTH HORMONE SECRETION IN CATTLE*

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Abstract: The lymphocytic growth hormone (GH) and nitric oxide (NO) levels were studied among three different age groups of cattle viz. cyclic adult cows (3-8 years of age), young female calves (2 weeks old) and growing female calves (6 months old). The effects of leptin, sodium nitroprusside (SNP) and N-nitro-L-arginine methyl ester (L-NAME) on the secretion of lymphocytic GH and NO were also studied. Lymphocytic GH in young calves (90.92±6.21 ng/12 million lymphocytes) and growing calves (86.15±9.94 ng/12 million lymphocytes) were significantly higher than in adult cows (59.42±3.92 ng/12 million lymphocytes) without any significant change in lymphocytic NO secretion with age. Incubation of lymphocytes with SNP could significantly increase lymphocytic GH secretion only in adult cows, while in 6 months old calves it significantly reduced the lymphocytic NO secretion. Thus suggesting that in young calves the secretion of lymphocytic GH is independent of leptin and NO dependent mechanism.

Key words: Growth Hormone, Lymphocyte, Cattle.

INTRODUCTION

The growth hormone (GH) exerts pleiotropic effects on virtually every organ systems in the body. GH has been reported to have effect on reproduction [1], immune system [2,3], nervous system [4], bones [5], cardiovascular system [6], skin [7] and body metabolism [8]. The physiological importance of interactions between immune and endocrine systems is increasingly being getting attention [9,10], however most of the underlying mechanisms in this cross-talk are still not clear. The proposed mechanism for these inter-system communications is the production of different neuropeptides and hormones from immune cells [11-13]. In addition to various neuropeptides and other classical pituitary hormones peripheral blood mononuclear cells (PBMCs); in the following will be addressed to as lymphocytes, produce GH [14,15]. The lymphocytic GH is largely similar to its pituitary counterpart [16]. Lymphocytes are also known to express leptin receptors and GH [14] and GH mRNA [17]. These hormones can bind to their specific receptors on immune cells to exert autocrine/paracrine effects.

GH is usually required for the timing of sexual maturity and puberty. Since delayed or absence of puberty is often associated with GH-deficient or GH-resistant states and GH administration accelerates puberty. For example, puberty is delayed in GH deficient women [18]. Puberty is similarly delayed in GH receptor (GHR)-knock out mice [19] and GH-
releasing hormone (GHRH)-immunised cattle [20]. The human lymphocytes express higher levels of growth hormone receptor (GHR) genes during pubertal period as compared to prepubertal and adult stages [21]. Previously, [14] reported that leptin induces GH from pig PBMCs via a protein kinase and nitric oxide (NO) dependent mechanism. Therefore, keeping in view the role of GH in development and puberty, the present study to explore the capacity of lymphocytes to secrete GH in pre-pubertal animals and mechanisms regulating the lymphocytic GH secretion was undertaken.

**MATERIAL AND METHODS**

**Animals:** The study was conducted on three different age groups of the animals. Group I comprised of 8 non-pregnant cyclic healthy Holstein-Friesian cows in the age group of 3-8 years. Group II comprised of 12 young female calves of about 2 weeks age. Group III comprised of 6 growing female calves of about 6 months age. Blood samples of 800 ml from adult and six months age group, and 200 ml from two weeks age group were collected in EDTA@ 2 mg per ml of blood by jugular vein puncture.

**Separation of lymphocytes:** The lymphocytes were separated as described previously [22,23]. The EDTA mixed blood was centrifuged at 2600 rpm for 20 minutes at 20°C, the buffy coat was harvested by pasteur pipette and resuspended in 1:2 v:v Hanks’ balanced salt solution (HBSS) (Sigma, Steinheim, Germany). Buffy coats were carefully layered on a lymphocyte separation medium, Lymphodex (Inno-Train, GmbH, Germany), at a concentration of 2:3 v:v and centrifuged at 2100 rpm for 30 minutes at 20°C to separate the lymphocytes. The ring of lymphocytes was sucked out, and the cells were resuspended and washed four times with HBSS. Contaminating erythrocytes were then lysed through a short incubation in double-distilled water and again washed with HBSS.

**Cell culture:** Lymphocytes were resuspended in a combination of 1:1 RPMI-1640 medium (Sigma, Steinheim, Germany) and HBSS solutions. The cell count was adjusted at 1×10^6 per ml, and subsequently incubated for 30 minutes at 38.6°C and then cell viability was tested by trypan blue exclusion test and cells showing more than 95% viability were cultured in three four well culture plates (Nunc Brand Product, Denmark) in RPMI and HBSS medium supplemented with 10% newborn calf serum (sigma Steinheim, Germany), 1% antibiotic/antimycotic mixture (Sigma, Steinheim, Germany) containing 10,000 IU penicillin, 10 mg streptomycin and 25 µg amphotericin per ml. The cells from these animals were incubated as control, or treated with leptin (100 nM), sodium nitroprusside (SNP) (1mM), leptin (100nM) plus SNP (1mM), N-nitro-L-arginine methyl ester (L-NNAME) (0.5 mM), for 72 hours at 38.6°C temperature and 5% carbon-dioxide (CO₂).

**Gel chromatography:** At the end of 72 hours of incubation, media from three plates (12 wells) of each treatment were pooled and centrifuged at 2160 rpm for 10 minutes at 20°C and after centrifugation one ml of pooled sample was stored at -20°C for nitric oxide measurement and remaining 11 ml for GH was eluted by gel chromatography using 1.5×30 cm Sephadex G-50 fine column. The column was equilibrated with 0.1M 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.

**GH assay:** GH was measured in duplicate by a homologous double anti-body radioimmuno assay (RIA) according to the method described by Bauer, M. and Parvizi, [24] and adapted for GH measurements in cell culture medium. Briefly, highly purified porcine GH was used as standard and for iodination. A highly specific anti-porcine GH antiserum was used as first anti-body and anti-rabbit GH anti-serum was used as second anti-body. This anti-serum shows no cross reaction with other adrenohypophysial hormones. All reagents were diluted in assay buffer. The lower limit of detection (10% displacement) was 0.5 µg/l. Half maximum displacement (ED₅₀) was achieved at 6 µg/l. The intra and inter-assay coefficients of variation were 7.5% and 15% respectively. GH was measured in all 26 aliquots of each treatment. For statistical evaluations GH in aliquots 7-16 were pooled.

**Nitric oxide (NO) measurement:** In aqueous solutions, that contain no heme protein, NO is oxidized to nitrite only [25], which can serve as an
indirect marker for the presence of NO [26]. Nitrite levels were measured in cell culture supernatants after conversion of nitrate in samples to nitrite using NADH-dependent nitrate reductase enzyme by an colorimetric assay based on Griess reaction as described by Dixit and Parvizi [12]. Briefly, all reagents were freshly prepared before assay and samples were incubated with freshly aliquoted beta-NADH and nitrate reductase (both reagents from Sigma) from Aspergillus sp. in 96- well plates for 16 hours. Total nitrite in the sample was assayed using equal amounts of sample and Griess reagent (1% sulfanilamide and 0.1% N-[1-naphthylene] ethylenediamine in 5% concentrated phosphoric acid). Amounts of nitrite were estimated from a standard curve of sodium nitrite, and absorbance was measured spectrophotometrically at 540 nm with an assay sensitivity of 1 µM.

**Stastical analysis:** The results were stastically analysed by ANOVA followed by t-test for comparision between groups and are expressed as mean ± SEM.

**RESULTS**

Lymphocytic GH secretion: Lymphocytic GH secretion in 2 weeks old female calves (90.92 ± 6.21 ng/12 million lymphocytes, Pd"0.01) and 6 months old calves (86.15 ± 9.94 ng/12 million lymphocytes) (Fig. 2).

Lymphocytic NO secretion: Among different age groups there was not much difference in lymphocytic NO secretion. (Table 1). In the 6 months old group leptin treatment did not induce any change in lymphocytic NO secretion. Surprisingly, incubation of lymphocytes with SNP (20.40±0.71 µM nitrite) and leptin plus SNP (21.32±0.64 µM nitrite) in this age group significantly (P<0.001) decreased the lymphocytic NO secretion when compared with control (30.55 ± 1.49 µM nitrite) (Fig. 3). In the 2 weeks old and adult cow groups, incubation of lymphocytes with L-NAME, leptin and SNP failed to induce any significant change in the lymphocytic NO secretion.

**DISCUSSION**

To our knowledge, the present study is the first to show a significant difference in lymphocytic GH secretion with advancement of age. Growth hormone and insulin-like growth factor-I (IGF-I) are endocrine regulators of growth and differentiation within multiple tissues [27]. Therefore, the GH and IGF systems are important components of nutrition, growth and reproduction [28]. In the present study the higher lymphocytic GH levels in 2 weeks and 6 months old calves than adult cyclic cows indicate a possible age dependent role before puberty. To our knowledge, no similar studies are available in literature. The lymphocytes derived GH binds to the lymphocyte GH receptors [29], indicating that it may act as an autocrine, paracrine or both fashions in vivo. Thus the variations in the lymphocytic GH secretion might be of significance, since lymphocytic GH might function as a cytokine [30], thereby suggesting that GH is intimately involved in lymphocyte function and expression of certain cytokines during critical period of fetal development [31], and thus also possibly in post-natal development and growth.

GH is required for timing of sexual maturity and puberty. Since delayed or absence of puberty is often

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<th>Cyclic adult cattle</th>
<th>Two weeks female calves (Young calves)</th>
<th>Six months female calves (Growing calves)</th>
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<tr>
<td>Lymphocytic GH (ng/12 million lymphocytes) Mean±SEM</td>
<td>59.42±3.92</td>
<td>90.92±6.21 ** vs. adult</td>
<td>86.15±9.94 * vs. adult</td>
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<tr>
<td>Lymphocytic NO (µM nitrite) Mean±SEM</td>
<td>29.07±1.97</td>
<td>27.45±0.97</td>
<td>30.55±1.49</td>
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Table 1: Lymphocytic Growth Hormone and Nitric Oxide secretion in different age groups in cattle (* P < 0.05; ** P< 0.01)
associated with GH-deficient or GH resistant states and GH administration accelerates puberty. For instance, puberty is delayed in GH deficient women [18]. Puberty is similarly delayed in GH receptor (GHR) knockout mice [19] and GH-releasing hormone (GHRH)-immunized cattle [20]. The human lymphocytes express higher levels of growth hormone receptor (GHR) genes during pubertal period as compared to prepubertal and adult stages [21]. Studies indicate that factors that compromise GH secretion during maturation may have a deleterious effect on growth and onset of fertility in female monkeys [32]. Hence we suggest that the lymphocytic GH might play role in puberty and sexual maturity in an autocrine/paracrine manner in the development and growth of various body organs including reproductive system.

Peripheral lymphocytes express leptin receptors and GH in pig [14] and GH receptors human being [33]. Bovine fetal lymphoid cells have also been reported to express GH receptors [31]. The percentage of PBMC expressing GH receptors has been found to be lower in older than in young human beings [34]. GH receptor deficient (GHRD) cattle have been shown to have impaired ovarian development [35]. These findings along with our study support that the higher lymphocytic GH secretion in young age in cattle may in an autocrine/paracrine manner help in the growth and development.

Leptin, an adipocyte derived polypeptide, plays important role in regulation of food intake and energy expenditure [36]. Leptin is involved in a variety of cellular functions, including the modulation of immune cell functions [37]. Sodium nitroprusside (SNP), a nitric oxide donor [38]. N-nitro-L-arginine methyl ester (L-NAME) is a nitric oxide synthase (NOS) inhibitor [14].

In our study, the incubation of lymphocytes with leptin insignificantly increased lymphocytic GH secretion in all the three different age groups of cattle without any effect of lymphocytic NO secretion. This finding is different from the previous result in pig, which indicated significant elevation in lymphocytic GH secretion through NO dependent mechanism [14]. Surprisingly, in our study, incubation of lymphocytes with SNP, a nitric oxide donor, significantly decreased the lymphocytic NO secretion in growing female calves. But it was insignificantly increased in adult cyclic cows. The decrease in lymphocytic NO secretion with SNP treatment probably might be due to rebound effect, the characteristic of a compound to produce reverse effects when either the effect of the compound has been passed or when subject no longer respond to the treatment compound. The lymphocytic GH was significantly (P≤0.01) increased when incubated
with SNP in adult cows. But in 6 months calves it was insignificantly increased. Incubation of lymphocytes with L-NAME in 2 weeks age group, lymphocytic GH was not changed, but lymphocytic NO was insignificantly decreased. The incubation of lymphocytes with L-NAME in adult cows insignificantly decreased the lymphocytic GH and NO secretion. Thus we suggest that secretion of lymphocytic GH is independent of NO secretion in 2 weeks and 6 months old calves, and it is different from the previous findings in pig, which suggested that lymphocytic GH secretion is dependent on mechanism involving leptin and NO [14]. But in adult cows it appears to be dependent on NO secretion. Role of leptin has been established in energy balance and food intake. Further it has been suggested that leptin act as a signal triggering puberty and sexual maturity [39]. Here we hypothesize that lymphocytic GH might have autocrine/paracrine and systemic actions in growth and puberty, but it is independent of mechanism involving leptin and/or nitric oxide.

In conclusion, higher level of lymphocytic GH secretion in pre-pubertal cow-calves indicates a possible age dependent role before puberty. This could be in an autocrine/paracrine and systemic manner in the development of various organ systems in the body. The mechanism of lymphocytic GH secretion in prepubertal animals is independent of leptin and nitric oxide dependent mechanism. Although the intracellular mechanism of action of lymphocytic derived growth hormone is still unexplained and further research may establish an important role of lymphocytic GH in pathophysiological conditions.

REFERENCES