Analysis of Major Isoforms of Human Growth Hormone before and after Intensive Physical Exercise

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We developed a semiquantitative method for the analysis of main growth hormone isoforms. The use of immunoaffinity sorbents, two-dimensional electrophoresis, and immunoblotting allows detection of more than 90% circulating growth hormone. It was demonstrated that the proportion of growth hormone isoforms in human serum before and after strenuous exercise remained unchanged.

Key Words: *growth hormone; physical activity; two-dimensional electrophoresis; immunoblotting*

Human growth hormone (GH) is encoded by gene *GH1*(NCBI GeneID: 2688) located in chromosome 17q22-24. GH is mainly produced by somatotropic cells of the pituitary gland and is secreted into the blood where it directly or through insulin-like growth factors participates in processes of growth and development [5].

GH is characterized by high molecular heterogeneity. There are several splice-isoforms of growth hormone (Fig.1). The principal isoform I (22 kDa) contains 191 amino-acid residues and two disulfide bridges [1,2]. This isoform constitutes 85% of all circulating GH. Isoform II (20 kDa) lacking fragment 32-46 (α-helix) as a result of alternative splicing of pre-mRNA constitutes about 10%. Removal of whole exon 3 yields isoform III (17.5 kDa, up to 4%).The content of isoforms IV and V with molecular weights of 11.3 and 3 kDa, respectively, in healthy human does not exceed 1%. In the blood, the major part of GH is bound into complexes with extracellular receptor fragment and also forms dimers and oligomers [1,9].

Physical exercises, stress and environmental changes stimulate growth hormone production [7,9]. The total blood concentration of GH depends on various parameters: age, physical state, environment, physical activity, lifestyle, *etc.* and varies from 0.1 to 30 ng/ml [6,12]. Different GH isoforms are characterized by different affinity to the receptor, which seems to determine their physiological effect on the target cells [8,11]. Selective and highly specific method of detection of different GH isoforms is required, because they little differ immunochemically from each other.

Current methods for measurement of GH concentration ELISA and radioimmunoassay [3,4,10] have sensitivity of 0.05-0.10 ng/ml. However, there are still no methods allowing simultaneous detection of all major GH isoforms and their proportions.

The objective of this study was to develop a method for evaluation of the major GH isoforms using two-dimensional electrophoresis and immunoblotting in serum samples from athletes before and after strenuous activities.

METHODS

GH isoforms were assayed in serum samples from 22 athletes: 16 men (M1-M16; 15-24 years, BMI 19.4-25.7 kg/m^2) and 6 women (W1-W6, 18-24 years, BMI 19.4-21.9 kg/m^2). All athletes gave

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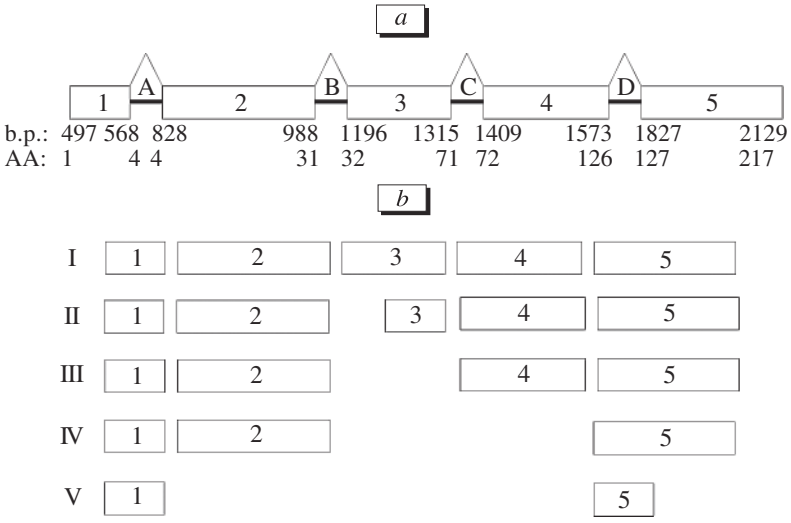

Fig. 1. Human GH. *a*) 1-5: exons, A-D: introns; *b*) I – 22 kDa (822 b.p.), II – 20 kDa (777 b.p.), III – 17.5 kDa (702 b.p.); IV – 11.3 kDa (537 b.p.), V – 3 kDa (376 b.p.). AA: amino acid.

informed concern for participation in this study. The study was approved by Ethical Committee of Russian Physical Training and Sports Institute.

All participants performed a loading test on a h/p Cosmos treadmill with stepwise increasing working power before noon (initial tape speed 2.5 m/sec, slope 1°, duration of each stage 3 min, speed increment 0.5 m/sec). Parameters of gas exchange, blood pressure, and heart rate were measured and peak oxygen consumption (POC) was estimated. Lactate concentration was measured during last 15 minutes of each step. The test was performed until the moment when the examinee failed to follow the tape speed.

Venous blood was sampled at rest before the test and immediately after the exercise. The serum was isolated and frozen at -20°C. Total GH concentration was assessed using EIA 1787 kit (DRG International Inc.) according to manufacturer's instructions with minor modifications.

For generation of polyclonal antibodies, rabbits were immunized with recombinant human GH (rGH was kindly provided by Prof. A. G. Gabibov). First immunization (1 mg rGH) was performed with complete Friend's adjuvant and subsequent immunizations (1 mg rGH) were performed in incomplete Friend's adjuvant. After 4 immunizations, the serum samples were pooled for affinity separation of antibodies.

rGH (2.25 mg/ml) was covalently immobilized on Aminolink sorbent (Pierce). This affinity sorbent was used for separation of specific antibodies against GH from the immune serum. Thereafter, affinity purified polyclonal antibodies were immobilized on SulfoLink sorbent (Pierce).

GH isolation from serum samples was performed on an affinity sorbent containing antibodies against rGH. The serum (1 ml) was passed 5 times through a column with 1 ml affinity sorbent. Nonspecifically bound proteins were removed by washing with PBS. GH was eluted with glycine pH 2.0 and after neutralization with Tris pH 9.0 was concentrated in Amicon Ultra-4 centrifuge tubes (Milipore) with regenerated cellulose (MWCO from 3000 to 50 μ).

Isoelectric focusing was performed on ElectrofaretIQ (Proteome Systems). The sample was applied on a 11-cm gel slab, pH 4.7-5.9 (BioRad) and focusing was carried out until attaining final voltage of 100,000 V/h. After equalization of proteins separated by isoelectric point, electrophoresis on ready-made 11-cm 8-16% gels (Proteome System) was performed. Semidry transfer was carried out on a Semi-Dry Trans Blott cell blotter (Bio-Rad).

Samples were transferred from gel to Immobilon-P PVDF membrane (Millipore). The membranes were treated with ECL Advance Blocking Reagent (GE Healthcare) and incubated with polyclonal rabbit antibodies against rGH (1 mg/ml) for 1 h in blocking solution. After washout, incubation with horseradish peroxidase-conjugated polyclonal goat anti-rabbit immunoglobulin (Sigma-Aldrich) was performed for 1 h. Detection of samples was carried out using chemiluminescent ECL Advance Western Blotting Detection Kit (GE Healthcare). Exposure varied from 5 to 120 sec.

RESULTS

Results of loading treadmill test with progressive power steps are presented in Table 1.

TABLE 1. Loading Testing Results (*M±m*)

Group	VO_{2max}, ml/min	VO_{2max}/kg (POC)	Lactate, mmol/liter
Men (M1-M16)	4.1	61.3	10.2
Women (W1-W6)	3.2	56.1	10.1

The dynamics of GH concentration before and after exercise test is presented on Fig. 2.

GH concentration at rest in men was lower than in women (0.1-0.3 and 1-3 ng/ml, respectively). High basel level of GH in M1, M11, M13, and W6 was probably related to hormone release not depending on physical exercise.

Significant increase in GH blood concentration was observed after exercise: to 25 ng/ml in men and up to 17 ng/ml in women. The relative increase was also more pronounced in men. These results agree with published data on the effect of physical activity on GH secretion into the blood [13]. GH release started 5-10 min after the start of physical activity and peaked after 15-30 min [5,8]. Loading test usually lasted for 12-18 min. Thus, GH concentration was measured at the peak of secretion. Wide range of GH values after exercise (3-25 ng/ml) confirms individual character of hormone response to physical activity.

For detection of GH isoforms in the serum, a new approach based on two-dimensional PAAG electrophoresis and immunoblotting with preliminary sample concentration on affinity sorbent was developed. To this end, affinity-purified polyclonal antibodies against human rGH were obtained.

For GH concentration, affinity sorbents containing covalently immobilized rabbit polyclonal antibodies were obtained. Antibody concentration varied from 0.1 to 3.0 mg/ml. The best results were obtained using sorbent 0.2 mg/ml. Sorbent capacity allowed isolation of GH from serum, where its concentration varied from 0.1 to 3.0 mg/ml [3,8]. The eluate was concentrated in Amicon Ultra-4 centrifuge tubes (Milipore). GH loss did not exceed 10% of total GH.

For isoelectric focusing, a narrow pH range was chosen (4.7-5.9; isoelectric point for rGH is 5.4), which increased resolution in the first direction and allowed separation of different posttranslational forms of 22-kDa isoform. Electrophoresis was carried out on gels with a density gradient of 8-16%, which increased the resolution in the zone of low molecular weights.

Optimal concentration of antibodies and conjugates was chosen in dot-blotting experiment. Dilutions 1:3000 (0.33 μg/ml) and 1:7500 (0.13 μg/ml) for antibodies and conjugates were used in all immunoblotting experiments.

For evaluation of the proportion of different GH isoforms, the dependence of signal intensity on rGH quantity was plotted. To this end, 5, 15, 25 and 40 ng/ml rGH was added to serum containing 0.1 ng/ml GH. The serum samples were processed by the standard protocol (Fig. 3).

The study demonstrated linearity of the method for GH concentration range of 5-40 ng/ml. Thus, this approach can be used for evaluation of the major GH isoforms in human serum.

Serum samples from athletes were analyzed by two-dimensional electrophoresis and immunoblotting. GH concentration before and after loading test was 0.5 and 15 ng/ml, respectively. The results of detection of serum chemiluminescence are presented on Figure 3 (exposure 60 sec). Signal intensity of 22 and 20 kDa isoforms was assessed using Quantity One software (Bio-Rad). The exposure was chosen so that the signal intensity of 22 kDa isoform was withing the measurement range. The ratio 22/20 kDa in samples before and after loading test was 10±2.

Analysis of affinity-separated GH samples failed to detect 17 kDa and smaller isoforms constituting less than 5% of serum GH. Detection sensi-

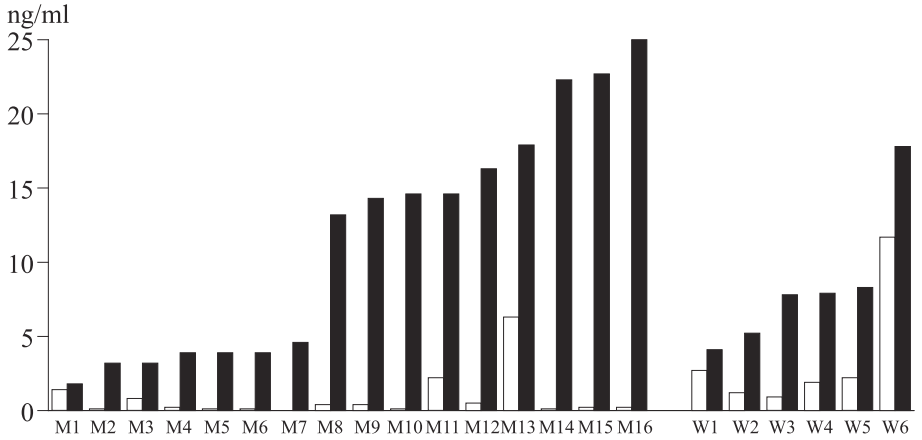

Fig. 2. GH concentration before (white) and after (black) loading test in men (M1-M16) and women (W1-W6).

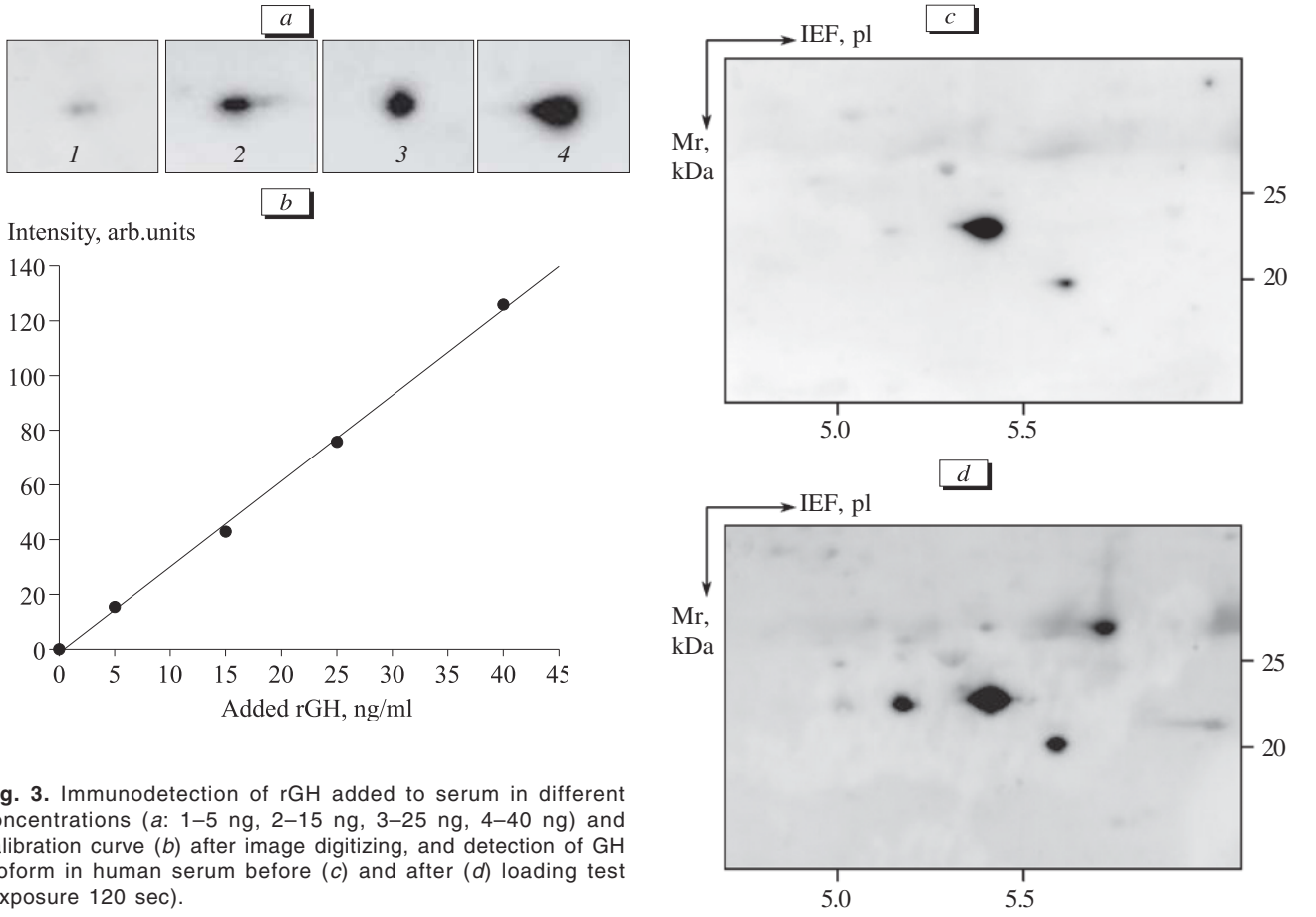

Fig. 3. Immunodetection of rGH added to serum in different concentrations (*a*: 1–5 ng, 2–15 ng, 3–25 ng, 4–40 ng) and calibration curve (*b*) after image digitizing, and detection of GH isoform in human serum before (*c*) and after (*d*) loading test (exposure 120 sec).

tivity and the effectiveness of GH separation should be increased for this analysis.

Thus, we developed a method for assay of GH isoforms in the serum of well-trained athletes and showed that the proportion between 22 and 20 kDa isoforms remained unchanged after increasing the total GH concentration and did not depend on physical activity.

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