Mass Spectrometric Profile of the Serum as a Marker of Experimental Psychoemotional Stress in Rats

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Mass spectrometric profiling of blood serum from stressed and control rats was used for the detection of stress marker proteins. Profiling with reversed-phase chips revealed 6 discriminatory mass spectrometry peaks. Cluster analysis showed that the animals can be divided into 2 groups by the intensity of these peaks. One group mainly consists of stressed rats, while other includes control specimens. Profiling of serum samples after fractionation on reversed-phase magnetic granules allowed us to identify an 8910-Da biomarker. Our results indicate that mass spectrometric profiling holds much promise for the detection of stress markers in the serum.

Key Words: *stress; mass spectrometry; biomarkers*

One of the major problems in the study of emotional stress is early diagnostics of the resistance or predisposition of subjects to stress exposure. A low-molecular-weight factor was isolated from the brain of stress-resistant rats [4]. This factor is relatively thermostable and is not hydrolyzed by pronase. Administration of the factor rats predisposed to emotional stress significantly increased the resistance to stress (*i.e.*, survival under conditions of experimental emotional stress).

Heat shock proteins (HSP) provide protection of cells and tissues under stress conditions [3,9]. One of the main properties of HSP is chaperone activity: they can bind and normalize function of conformationally modified proteins. The family of HSP70 was extensively studied [3,10]. It was hypothesized that HSP70 expression induces the temporal resistance to stress, protects cell proteins from damage, and stimulates cell reparation. These changes are accompanied by an increase in serum content of anti-HSP70 antibodies [5,9].

Previous studies revealed conformational changes in blood albumins of stressed rats (particularly in animals predisposed to emotional stress) [6].

These data suggest that the blood of stressed animals contains specific protein factors serving as stress marker. Time-of-flight mass spectrometry is a modern high-efficiency method of proteomic studies [2]. This method is extensively used to identify biomarkers of various diseases and physiological states in blood serum [7,8].

Here we studied changes in the mass spectrometric profile of blood serum from stressed rats, identified marker mass spectrometry peaks, and developed a model system for the diagnostics of stress by mass spectra.

MATERIALS AND METHODS

Experiments were performed on 80 male Wistar rats (220.0±5.2 g) in the autumn-winter period. The animals were quarantined in cages (10 specimens per cage) for 10 days before the start of the study. They had free access to water and food.

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Our experiment was conducted according to the rules for studies involving experimental animals (order of the Ministry of Health of the USSR, No. 1179, 11.10.1983; and order of the Russian Ministry of Health, No. 267, 19.06.2003).

Immobilization in combination with threshold electrocutaneous stimulation of animals served as a model of acute emotional stress. The rats were immobilized in individual Plexiglas cages (160×65×50 mm). Electrocutaneous stimulation with threshold current (4-6 V, 50 Hz, pulse duration 1 msec) was applied to the tail. The length of stochastic stimulation was 30 sec or 1 min. The total time of stimulation was 1 h. The control group consisted of 40 unstressed rats.

The animals were sacrificed. Stress marker organs (thymus and adrenal glands) were isolated and weighted, blood for biochemical analysis was obtained. The serum was separated routinely by centrifugation of the blood after clotting. The serum from stressed and control rats was analyzed by a blind method.

Mass spectrometric profiling of serum samples was performed by the following two methods: mass spectrometry of protein chips SELDI-TOF (Surface-enhanced laser desorption/ionization time-of-flight) and technology of magnetic granules ClinProt. Both methods include the stage of chromatographic fractionation of biological samples before mass spectrometric study. The SELDI-TOF technology suggests fractionation on protein chips with various types of the chromatographic surface [1]. The ClinProt technology is based on incubation of the serum with magnetic granules of different chromatographic surface.

Serum samples from stressed (n=20) and control rats (n=20) were profiled on chips with a reversed-phase chromatographic carrier (H4, Ciphergen Biosystems) using a SELDI-TOF mass spectrometer. Washing and binding were performed with a working solution of 50 mM phosphate buffered saline, which contained 100 mM NaCl, 10 vol % acetonitrile, and 0.1 vol % trifluoroacetic acid (acetonitrile and trifluoroacetic acid of HPLC purity, Merck). Chip spots were pretreated with 5 μl acetonitrile. Chips were combined in a 96-well bioprocessor (Ciphergen Biosystems). A working buffer (350 ml) was added to each well. Incubation was performed for 5 min with thorough shaking. The buffer was removed. The serum (100 μl) was diluted in a working buffer (1:20) and added immediately. The samples were removed after 30-min incubation at room temperature with thorough shaking. The wells were washed 3 times with 350 μl working buffer (5-min washing). The chips were removed from a bioprocessor, washed 5 times with deionized water, and dried on air. The matrix solution ($^1/_5$ dilution of a saturated solution of α-cyano-4-hydrocinnamic acid in 50% acetonitrile and 0.5% trifluoroacetic acid) was layered in 2 portions of 0.5 μl. The chip was dried in air and put in a Protein Biology System II SELDI-TOF mass spectrometer (PBS II, Ciphergen Biosystems). Mass spectra of compounds (7-60 kDa) were obtained automatically (laser intensity 220; detector sensitivity 9; 90 laser pulses per chip spot).

In a special series, serum samples from stressed (n=20) and control rats (n=20) were incubated with C8 reversed-phase magnetic granules (ClinProt, Bruker Daltonics) according to the manufacturer's recommendations. The solution eluted from magnetic granules (1 μl) was placed on a gold chip (PBS II mass spectrometer-compatible MALDI target). The spots were dried and treated 2 times with 0.5 μl matrix solution. A saturated solution of α-cyano-4-hydrocinnamic acid (Bruker Daltonics) in 50 vol % acetonitrile containing 0.5 vol % trifluoroacetic acid was 2-fold diluted was the same solvent and served as a matrix. Profiling was performed on a PBS II mass spectrometer. The spectra were recorded automatically under the following protocols: optimized protocol for peptides and low-molecular-weight proteins (up to 20 kDa); and optimized protocol for 6-60-kDa proteins. Laser intensity was 190 and 195, respectively (detector sensitivity 8; 90 laser pulses per spot). The spectra of each sample were recorded in at least two repetitions to obtain reproducible results.

The spectra were calibrated with external standards, which contained a mixture of proteins of known mass (Bruker Daltonics). The accuracy of mass measurement was at least 0.1%.

The derived mass spectra were statistically analyzed by means of Protein Chip and Biomarker Wizard Software with default parameters (Ciphergen Biosystems). The spectra were normalized by intensity using the total ion current with a mass

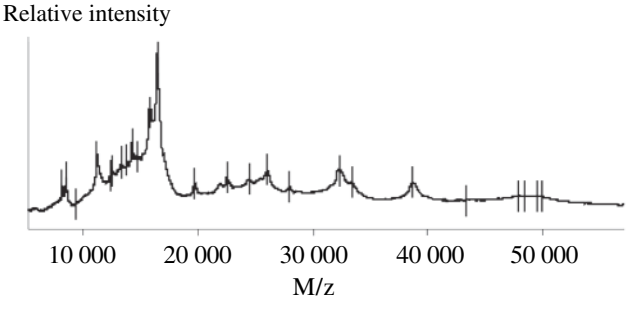

Fig. 1. Typical SELDI-TOF mass spectrum of rat serum on a H4 reversed-phase chip. Vertical marks: molecular weights determined by Biomarker Wiszard software for a comparative study.

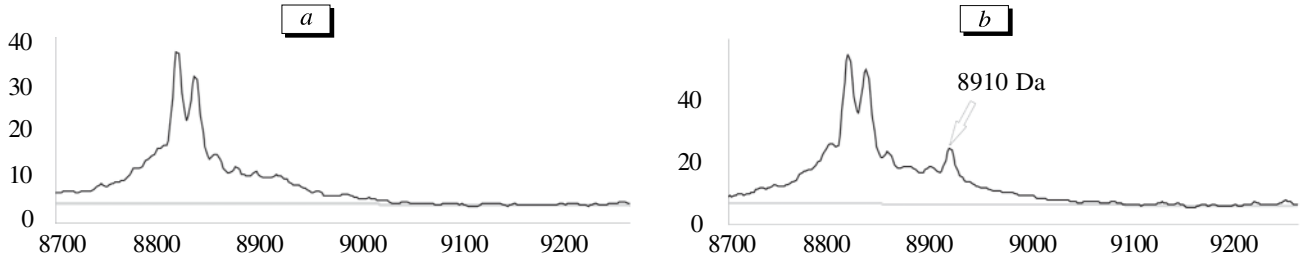

Fig. 2. Marker peak at the mass/charge ratio of 8910 Da. Mass spectra were obtained with reversed-phase magnetic granules. Control (*a*) and stress (*b*).

range of 7-60 kDa. Biomarker Wizard software (Student's *t* test) was used to estimate the mass/charge ratios (M/z), which differed in signal intensity during a comparative study of samples (discriminatory values of M/z).

The objects were clustered by discriminatory values of M/z using SPSS 11 Demo software (SPSS Inc.). Cluster analysis was performed with a squared Euclidean distance matrix. Dendrograms were calculated by the Ward method.

RESULTS

The state of stress in animals was estimated from a changes in the weight of marker organs (adrenal glands and thymus). The weights of the adrenal glands and thymus in control rats were 6.9±0.3 and 64.6±1.8 mg/100 g body weight, respectively. The weights of the adrenal glands and thymus in stressed rats (7.6±0.8 and 46.5±3.8 mg/100 g body weight, respectively) significantly differed from that in control animals ($p<0.05$), which serves as a criterion of stress.

Proteomic profiling of serum samples from these animals on reversed-phase chromatographic chips included the evaluation of significant differences in intensity of 30 individual values in the range of 7.5-55 kDa (automatic identification by means of Biomarker Wizard software, Fig. 1). The differences were significant at a significance level ≤1% (Student's *t* test).

Comparative study of the control and stressed rats revealed 6 discriminatory marker M/z values in the range of 10.5-38.2 Da. It should be emphasized that the intensity of marker peaks increased after stress exposure. The accuracy of mass measurement was 0.1%. The intensity is presented in relative units. M/z values of 19,085 and 38,121 Da served as the most reliable markers of emotional stress (Table 1). It should be noted that proteins tend to form monovalent and polyvalent ions in MALDI. For example, the bivalent ion of a 20,000-Da compound will have the M/z value of 10,000 Da. Taking into account these data and error of protein mass measurement, it may be suggested that M/z of 19,085 Da corresponds to the bivalent ion of a 38,121-Da protein.

Cluster analysis of serum samples was performed by the intensity of 6 marker values of M/z. The samples were arranged in 2 large clusters: cluster I, 3 experimental samples of 18 samples (control cluster); and cluster II, 5 control samples of 22 samples (stress cluster). Proteomic profiling of serum samples allowed us to distinguish the groups of control and stressed animals.

Profiling of the serum with magnetic granules was performed on another sample of 20 stressed rats and 20 control animals. Mass spectrometry peaks were identified automatically. Significant inter-

TABLE 1. Marker Mass Spectrometry Peaks during Profiling of Rat Serum on H4 Reversed-Phase Chips

M/z of a peak, Da	Significance level, Student's *t* test	Mean peak intensity	
		control	stress
10,589	0.007	2.29±0.51	2.70±0.36
12,712	0.008	0.38±0.31	0.59±0.32
12,776	0.009	0.25±0.32	0.48±0.37
19,085	0.0001	0.43±0.20	0.74±0.20
25,404	0.007	1.00±0.38	1.35±0.24
38,121	0.002	0.66±0.28	1.00±0.29

group differences in intensity were found only for one peak with M/z of 8912.2 Da (significance level < 1%, Student's *t* test). The intensity of this peak in serum samples from stressed rats was higher than in control animals. Characteristic mass spectra are shown in Fig. 2. An 8904.9-Da peak on the spectra of medium-molecular-weight proteins probably corresponds to the protein, which is characterized by an 8912.2-Da peak on the spectra of low-molecular-weight proteins. Small differences in the weight are within instrumental error.

Blind study of blood serum from control and stressed rats was performed by 2 methods and revealed the presence of marker peaks for the state of stress. Cluster analysis showed that serum samples may be classified into stressed and control groups by the intensity of marker peaks. Our results indicate that that mass spectrometric profiling holds much promise for the detection of stress markers in blood serum.

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